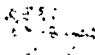
PROCEEDINGS OF THE NINTH ANNUAL CONFERENCE

ON

THE NEPHROTIC SYNDROME

EDITED BY

JACK METCOFF, M.D.



HELD AT THE NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND
OCTOBER 24–26, 1957

SPONSORED BY THE NATIONAL NEPHROSIS FOUNDATION, INC.
143 E. 35TH ST., NEW YORK, N. Y.

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INTRODUCTION

The Ninth Annual Conference on The Nephrotic Syndrome sponsored by the National Nephrosis Foundation convened at one thirty-eight o'clock at the National Institutes of Health, Bethesda, Maryland, Drs. James Baxter and Howard Goodman, Laboratory of Metabolism, National Institutes of Health, hosts.

DR. BAXTER: We are glad to see you here and to welcome you on behalf of those of us at the NIH. These sessions are informal, with emphasis on discussion, as in the previous conferences.

I. ANTIGENS, ANTIBODIES AND RENAL LESIONS

DR. HOWARD GOODMAN (National Heart Institute): Perhaps the weather has held up Dr. Heymann. (Dr. Heymann entered the meeting.) It could not be a better time, Dr. Heymann. Will you come in and make yourself comfortable, then let me ask you to chair this afternoon's meeting. Dr. Heymann!

(Dr. Walter Heymann, Western Reserve University School of Medicine, Department of Pediatrics, assumed the chair.)

CHAIRMAN HEYMANN: I usually am on time; but I thought that one-thirty p.m. was scheduled so that we would all be here by two o'clock. (Laughter) Thus I thought I would be fifteen minutes early. I apologize for being late just the same and quote as an excuse that we drove from Cleveland.

This afternoon, we have a session on antigens, antibodies, and renal lesions produced by anti-kidney sera.

The program is crowded. If it is all right with all of you, we could take the two first presentations and have a joint discussion at the end of the second paper.

Dr. Seegal, would you like to start?

A. Localization of Rabbit and Duck Anti-kidney Antibodies

DR. BEATRICE C. SEEGAL (Department of Microbiology, College of Physicians and Surgeons, New York): I should like to thank you for the invitation to speak here. It is not very often, in this specialized field, that it is possible to put one's results before such a critical audience. We value the opportunity to report on our studies in experimental nephrosis and nephritis.*

Several people have participated in the experiments to be described. Dr. Margaret Bevans has reviewed the renal lesions and also initiated the use of the Coons' technique for studying the localization of nephrotoxic antisera. Dr. Hsu who is searching for the antigen responsible for stimulating the production of nephrotoxic antiserum, has taken the microphotographs you will see. Dr. Triedman and Dr. Metzger have prepared the anti-rat-lung sera to be described. Mrs. Hasson, Mrs. Rothenberg and Dr. Urguhart have contributed in many ways to the project.

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*This work has been supported by grants from the National Science Foundation and The National Institutes of Health.

Our purpose has been to seek some information concerning the mechanism of experimentally produced nephritis and the nephrotic syndrome. The natural history of the renal disease induced by the injection in rats of anti-rat-kidney sera is variable. There may be an immediate onset of a severe nephrotic syndrome characterized by marked proteinuria, hypercholesterolemia and edema. Death may occur in a week or two or the animal may live to develop chronic glomerulonephritis, dying months later of renal failure. On the other hand the initial nephrotic syndrome may be absent even though the renal disease also starts immediately following injection. Finally nephritis may develop only after a latent period of days or weeks. Both these latter two types of disease usually progress to chronic nephritis and terminate in renal failure months after the injection of the antiserum.

Both in vitro and in vivo studies of the reactions between nephrotoxic sera and rat tissues have been made in an attempt to obtain information concerning the cause of the variations in the progression of the renal disease. Some of the methods employed are of importance to the interpretation of the results. The antisera were prepared by injecting either rabbits or ducks with rat kidney or other rat organs (See Table 1). Both species of animals received prolonged immunization, which lasted three to four months when the organs were incorporated in Freund's adjuvant[1] and five to six months when saline suspensions were injected. Pooled sera obtained from two or more rabbits or ducks and first absorbed with rat red blood cells were used for injection into rats. Some ducks as well as rabbits provided pools of antisera which elicited an immediate nephrotic syndrome. Other duck and rabbit antisera produced immediate nephritis but no nephrotic syndrome. Still other pools of duck serum produced nephritis only after a latent period.

The organ antisera first were tested in vitro by the Ouchterlony agar diffusion technique[2] which revealed that all the organ antisera possessed many antibodies in common and some which were unique for the specific tissue. It was not possible to identify the antibody associated with nephritis. Further in vitro studies employed the Coons' technique[3]. In this aspect of the work fresh sections of normal kidney were covered with anti-rat-kidney serum. After 30 minutes, to allow for the binding of antibodies by the kidney antigens, the excess antiserum was washed off and an antibody to the antibody, namely duck anti-rabbit-globulin or rabbit anti-duck-globulin, tagged with fluorescein-isocyanate was added to the tissue. Wherever the anti-rat-kidney serum had been bound in the kidney parenchyma the fluorescent-antibody also was bound. When examined under ultraviolet light this site was recognized by its yellow green fluorescence. Here again there proved to be many antigens in the kidney which reacted with the antiserum. The nephrotoxic serum was bound to every part of the kidney when applied in high concentration. On addition of the fluorescent anti-rabbit-globulin or anti-duck-globulin the entire section fluoresced. Serial dilutions of the

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[1] Freund, J., Lipton, M. M., and Thompson, G. E., Aspermatogenesis in the Guinea Pig Induced by Testicular Tissue and Adjuvants, J. Exper. Med. 97: 711, 1953.

[2] Ouchterlony, O., Gel diffusion methods for immunological analysis, Sixth Internat. Cong. Microbiol., 2: 276, 1953.

[3] Coons, A. H., and Kaplan, M. H., Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody, J. Exper. Med., 91: 1, 1950.

anti-rat-kidney serum were added to sections of normal rat kidney and it was found that at dilutions of 1:30 to 1:80 antibodies to cells were no longer demonstrable but the basement membranes of tubules and glomeruli still bound antibody. When the nephrotoxic antiserum was diluted about 1:100 or 1:125 a point was reached where antibody was demonstrable only in the basement membranes of the glomeruli. Thus it appeared that antibodies specific for the basement membranes of the kidney could still be demonstrated at dilutions so great that other antibodies were lost from sight.

These studies using the Coons' technique to trace the binding of anti-kidney antibody by specific structures in the kidney were encouraging. They could not, however, answer the questions: What happens to anti-kidney or other anti-organ sera when injected into the living animal? Are the antisera bound in the glomeruli? Where in the glomeruli and in what quantity? Are they bound elsewhere? How long do they remain in the tissues?

TABLE 1

ANTI-RAT-ORGAN SERA INJECTED IN RATS

Localization in Glomeruli as Demonstrated by the Coons' Technique

Serum injected	Amount ml.	Number of rats	Nephritis produced	Day killed	Serum in glomeruli
RARKS	0.7-1.5	13	Immediate	4-291	Yes
DARKS	0.6-1.5	8	Immediate or delayed	1-17	Yes
RARPS	0.8, 0.9	2	Immediate	2, 3	Yes
DARLS	1.0-1.5	19	Immediate or delayed	1-259	Yes
RARAS	1.4, 1.5	2	None	3, 40	Yes
DARAS	1.3	2	None	1, 17	Yes
RARBS	1.5	2	None	3, 40	No
RARSS	1.5	2	None	3, 40	No
NRS	1.5	2	None	2, 16	No
NDS	1.2-1.5	4	None	2-24	No

RARKS Rabbit anti-rat-kidney serum
DARKS Duck anti-rat-kidney serum
RARPS Rabbit anti-rat placenta serum
DARLS Duck anti-rat-lung serum
RARAS Rabbit anti-rat-aorta serum
DARAS Duck anti-rat-aorta serum
RARBS Rabbit anti-rat-brain serum
RARSS Rabbit anti-rat-serum serum
NRS and NDS Normal rabbit or duck serum

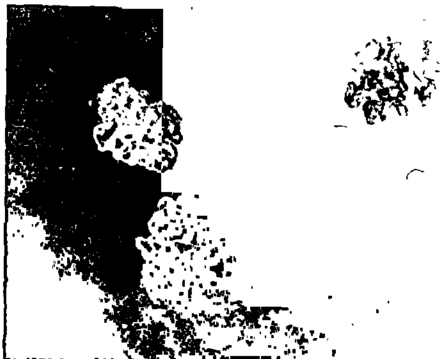


Fig. 1. Section of kidney from rat SDB707, killed 4 days after last of 3 daily injections, totaling 0.9 ml. of rabbit anti-rat-kidney serum. Section treated with fluorescein-tagged duck anti-rabbit-globulin and viewed by ultraviolet light. Only glomeruli have retained the fluorescent material, indicating localization of injected anti-renal antibody in this area (x 250).



Fig. 2. Section of kidney from rat SDB817, killed 6 days after last of 3 daily injections, totaling 1.2 ml. of duck anti-rat-lung serum. Section treated with fluorescein-tagged rabbit anti-duck-globulin and viewed by ultraviolet light. Only glomeruli have retained the fluorescent material, indicating localization of injected anti-lung antibody in this area (x 250).

In an attempt to follow the distribution in vivo of nephrotoxic serum, rats have been injected with anti-rat-kidney serum prepared either in the rabbit or duck, and with rabbit anti-rat-placenta serum and duck anti-rat-lung serum, all of which produced nephritis. Rabbit and duck anti-rat-aorta sera, rabbit anti-rat-brain serum, rabbit anti-rat-serum serum and normal rabbit and duck serum have been injected into control rats. Daily samples of urine have been tested for protein, the animals have been inspected for edema and ascites and have been weighed each day. Some of the pertinent data are summarized in Table 1.

One day to eight months after injection of the antisera the rats were sacrificed and sections of organs were frozen and stored in a CO_2 chest until cut in a cryostat according to Coons' technique. So far kidneys have been examined for the presence of the injected antisera in all animals, the lungs, spleen and adrenals in about half of the animals and the liver, heart, lymph nodes, thyroids, pancreas and ovaries in a few of the rats. The tissues may be kept in the CO_2 box for at least six months without showing any loss in content of the injected antiserum. Sections of the tissues are cut at 2 or 4 microns. They are placed in 95 per cent alcohol for 30 seconds which helps to fix them to the glass slide. After washing with buffered saline, fluorescein tagged duck anti-rabbit-globulin or rabbit anti-duck-globulin is added to the slides for 30 minutes. The sections are washed and covered with buffered glycerine and are ready for examination in ultraviolet light. Duplicate sets of tissue fixed in Zenker's fluid are stained with hematoxylin and eosin and periodic acid Schiff reagent.

The foreign serum was found in the basement membranes of the glomeruli in the kidneys from all the rats which were injected with a nephrotoxic antiserum, namely anti-kidney serum, anti-placenta serum or anti-lung serum. Figure 1 illustrates a kidney section from rat SDB 707 injected intravenously with pool 465-466, rabbit anti-rat-kidney serum. A total of 0.9 ml. was given on three successive days. After the first injection there was marked proteinuria and a full-blown nephrotic syndrome developed. When killed on the fourth day after injection the animal had gained 33 per cent in body weight. The urine contained 4 gm. per cent protein. There was 8 ml. of ascitic fluid in the peritoneal cavity. The serum cholesterol was 350 mg. per cent (75 mg. per cent is normal for the rat) and the blood urea nitrogen was 52 mg. per cent. It may be seen from Figure 1 that the specific fluorescence within the glomeruli is brilliant and there is no fluorescence in other areas of the kidney.

Figure 2 is a photomicrograph of a kidney from rat SDB 817 which was injected with 0.9 ml. of duck anti-rat-lung serum. This rat also had edema and when sacrificed six days following injection there were 5 ml. of fluid in the peritoneal cavity. Blood chemistries are not yet completed. When sections of the kidney were "stained" with rabbit anti-duck-globulin tagged with fluorescein isocyanate the duck serum was shown to localize in the kidney in a manner comparable to that seen following the injection of specific anti-kidney serum. The lung contained no demonstrable duck protein nor has duck protein been found in the lungs of any rat injected with duck anti-rat-lung serum.

Certain cells in the red pulp of the spleen and cells lining the capillaries of the adrenal cortex have contained the injected foreign serum. Figure 3 is a section of spleen from rat SDB 817. Fluorescence is seen in many cells outside the follicles and occasionally in cells within the follicles.



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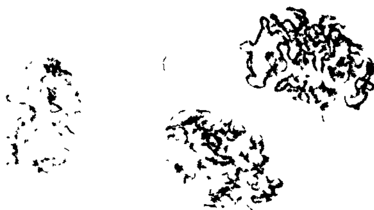
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Fig. 3. Section of spleen from rat SDB817, see Fig. 2. Section treated with fluorescein-tagged rabbit anti-duck-globulin and viewed by ultraviolet light. Some cells in red pulp have retained the fluorescent material, indicating that they also constitute a site for localization of injected anti-lung globulin (x 250).

Nephrotoxic sera are retained in glomeruli for months. This is illustrated in Figure 4 which is a photomicrograph of a kidney from rat H 851, killed 259 days following injection of 1.5 ml. of duck anti-rat-lung serum. The injected duck protein is demonstrable in the severely damaged glomeruli. One rat injected with rabbit anti-rat-kidney serum, which was killed 291 days later when dying of advanced chronic nephritis, presented a comparable picture. These findings confirm and extend the observations of Mellors and his associates[4] who have reported the presence of nephrotoxic sera in the glomeruli of rats over a period of three months.

Tissues from rats injected with nephrotoxic sera and stained with hematoxylin and eosin or Schiff's reagent have shown lesions only in the kidneys. The typical lesions of acute, subacute and chronic nephritis have been frequently described[5-7]. The lesions produced by anti-lung sera were similar to those produced by anti-kidney sera[8, 9].

The data so far presented have indicated a close correlation between localization in glomeruli of the injected anti-organ serum and the occurrence of nephritis. This has been further supported by the finding (Table 1) that neither anti-rat-brain nor anti-rat-serum serum localized in the glomeruli. Normal rabbit and duck sera also failed to show any localization in this area. Anti-rat-aorta serum proved to present a problem. Both ducks and rabbits were immunized with rat aorta. No renal pathology resulted from the injection of these sera. Nevertheless the examination of the kidneys from two rats injected with rabbit anti-aorta serum and two rats injected with duck anti-aorta serum showed localization in the glomeruli of the injected rabbit or duck antigen. The kidneys were studied from 1 to 64 days after injection. Fluorescence was bright only in the kidney which was taken from an animal receiving its last of three consecutive injections 1 day previously. Fluorescence was slight in the other sections. This observation showed that some sera which did not produce nephritis nevertheless could localize in the glomeruli to some extent.

It became imperative to find a method of evaluating the relation between the amount of anti-organ sera localized in glomeruli and the development of nephritis. A modification of the standard technique employed to demonstrate the specificity of the reaction between glomeruli and fluorescein tagged antiserum is being used to this

[4] Ortega, L. G., and Mellors, R. C., Analytical pathology. IV. The role of localized antibodies in the pathogenesis of nephrotoxic nephritis in the rat. J. Exper. Med. 104. 151, 1956.

[5] Heymann, W., Lund, H. Z., and Hackel, D. B., The Nephrotic Syndrome in Rats, with Special Reference to the Progression of the Glomerular Lesion and to the Use of Nephrotoxic Sera Obtained from Ducks. J. Lab. & Clin. Med. 39: 218, 1952.

[6] Ehrich, W., Forman, C. W., and Seifer, J., Diffuse Glomerulonephritis and Lipid Nephrosis. Arch. Path. 54: 463, 1952.

[7] Seegal, B. C. and Bevans, M., The Production of Glomerulonephritis by Immulogic Methods. J. Chron. Dis. 5: 153, 1957.

[8] Baxter, J. H., and Goodman, H., Nephrotoxic Serum Nephritis in Rats. I. Distribution and Specificity of the Antigen Responsible for the Production of Nephrotoxic Antibodies. J. Exper. Med. 104: 467, 1956.

[9] Triedman, R. S., and Metzger, H., Personal communication.

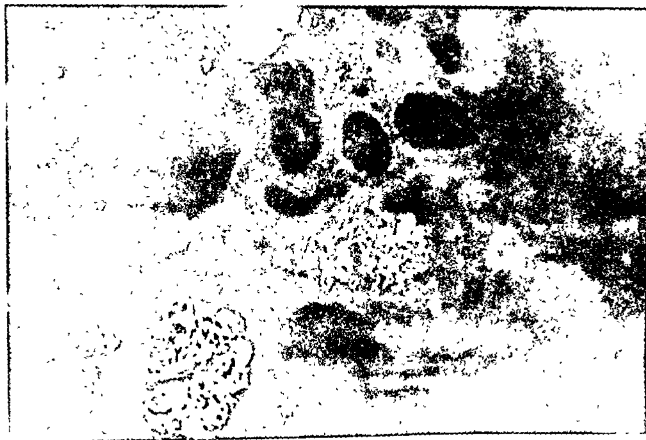


Fig. 4. Section of kidney from rat H851, killed 259 days after last of 3 daily injections, totaling 1.5 ml. of duck anti-rat-lung serum. Section treated with fluorescein-tagged rabbit anti-duck-globulin and viewed by ultraviolet light. Sclerosed glomeruli fluoresce although less brightly than did glomeruli from animals sacrificed earlier. It may be concluded that duck anti-lung globulin rains in glomeruli 8 months after injection (x 250).

end. The experiments which have been completed to date are few in number but indicate that the method offers a simple means of obtaining a rough estimate of the relative amount of foreign protein bound in a given area of the body. The technique may be illustrated by presenting a protocol of a specific animal. Rat SDB 963 was injected on two successive days with a total of 0.6 ml. of rabbit anti-rat-kidney serum. The animal developed a nephrotic syndrome. In three days the rat was killed and a piece of kidney frozen and sectioned. Two-fold dilutions of duck anti-rabbit-globulin were added to slides containing the kidney sections, and buffered saline to a control section. After 30 minutes all slides were washed in buffered saline and then covered for 30 minutes with duck anti-rabbit-globulin tagged with fluorescein isocyanate. On examination of these sections by ultraviolet light it was seen that the control section contained brightly fluorescent glomeruli, Figure 5. There was a barely perceptible decrease in brightness of fluorescence in the section previously treated with duck anti-rabbit globulin diluted 1:20. In the sections treated with 1:10, 1:5 and 1:2.5 dil
ness of fluorescence was found to be progressively less
section treated with the 1.5 dilution. Undiluted duck
the rabbit antigen in the glomeruli so that no fluorescence developed on addition of the tagged antiserum, Figure 7. It was concluded that the glomeruli in this rat retained so much of the injected rabbit antibody to rat kidney that undiluted duck anti-rabbit-globulin was required to "neutralize" this rabbit antibody and prevent it from binding the fluorescein tagged duck anti-rabbit-globulin.

Results obtained from the examination of kidneys from 28 rats tested by this "neutralization" technique are given in Table 2. It may be seen that kidneys from rats which were killed within three weeks after injection of rabbit anti-rat-kidney serum required either undiluted anti-rabbit serum or serum diluted 1:2.5 to cover the rabbit antibody in the glomeruli and prevent binding of the tagged anti-rabbit serum. After months of nephritis the amount of rabbit protein in the glomeruli has decreased since dilutions of untagged anti-rabbit serum varying from 1:5 to 1:20 neutralized the rabbit antibody in the glomeruli.

The injection of nephrotoxic sera prepared in the duck, namely anti-rat-kidney serum and anti-rat-lung serum produced similar results provided that the injection of these antisera were followed by the immediate onset of nephritis. The five rats injected with duck anti-rat-kidney serum, Pool III or Pool C, two antisera which produce nephritis only after a latent period, were killed within three weeks of injection. The addition of dilutions of duck anti-rabbit-globulin diluted 1:5 or 1:10 to sections of these rats' kidneys was adequate to prevent subsequent binding of duck anti-rabbit-globulin tagged with fluorescein isocyanate.

The four rats injected with rabbit or duck anti-aorta serum developed no nephritis. Nevertheless the injected antibody was found in the glomeruli. It may be seen from Table 2 that it did not persist as in the case of rats injected with nephrotoxic antiserum. After 64 days very little of the serum prepared in the rabbit remained and a 1:40 dilution of untagged duck anti-rabbit-globulin prevented binding of the tagged anti-rabbit serum.

From the results of the neutralization tests reported in Table 2 it appears probable that information concerning relative amounts of nephrotoxic serum localized in glomeruli, following the injection of different antisera, can be obtained. Furthermore the rate

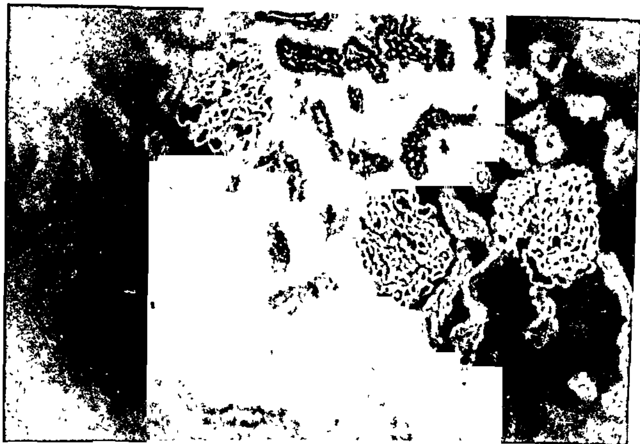


Fig. 5. Section of kidney from rat SDB963, killed 3 days after 2 successive daily injections, totaling 0.6 ml. of rabbit anti-rat-kidney serum. Section treated with fluorescein-tagged duck anti-rabbit globulin, and viewed by ultraviolet light. Only glomeruli have retained the fluorescent material (x250).

TABLE 2

ANTI-RAT-ORGAN SERA INJECTED IN RATS

Amount of Antibody to Rabbit or Duck Globulin Required to "Neutralize" the Anti-organ Sera Bound in the Glomeruli

Serum injected*	Number of rats	Nephritis produced	Day killed	Anti-rabbit or duck serum to "neutralize"
RARKS	5	Immediate	3-20	Undil. or 1:2.5
RARKS	7	Immediate	22-291	1:5 to 1:20
DARKS	2	Immediate	1, 15	Undil., 1:2.5
DARKS	5	Delayed	1-18	1:5 or 1:10
DARLS	2	Immediate	1, 10	1:2.5
DARLS	3	Immediate	70-259	Undil., 1:5, 1:10
RARAS	2	None	3, 17** 64	1:10, 1:20, 1:40
DARAS	2	None	1, 18	Undil., 1:10

*For "Serum injected" see Table 1 footnote.
**Left kidney removed 37 days before death.

loss of such antisera from the glomeruli can be estimated. It is possible that some correlation between nephritis and the amount and persistence of foreign protein in the glomeruli can be established.

The data presented here may be briefly reviewed. The studies described have demonstrated that there is variation in the pattern of response to the injection in rats of nephrotoxic sera. The disease produced may be associated with the immediate onset of the nephrotic syndrome and end in death of the animal within a few weeks or at most a few months; it may have an immediate onset but a more protracted course and finally it may not start for days or weeks following injection of the antiserum, although once initiated the disease usually ends in renal failure.

In vitro tests of nephrotoxic antisera have indicated that many antibodies are produced following immunization of rabbits or ducks with rat organs but the antibody responsible for nephritis is not identifiable by the Ouchterlony agar diffusion technique or by the Coons' technique.

When animals injected with nephrotoxic sera are sacrificed days or months later the injected antiserum is demonstrated by the Coons' technique in the glomerular membranes.

An immunologic method has been described for assessing the relative amount of the antiserum in the glomeruli. This value has varied with the antisera injected. Those

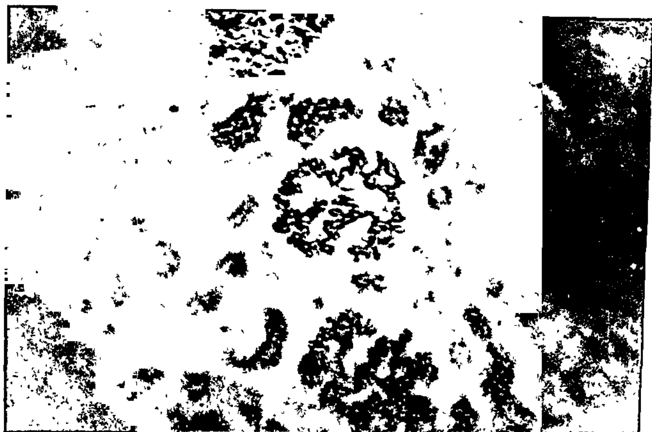


Fig. 6. Section of kidney from rat SDB963, see Fig. 5, first treated with untagged duck anti-rabbit-globulin, diluted 1:5 with buffered saline, then with fluorescein-tagged duck anti-rabbit-globulin. Viewed by ultraviolet light the glomerular fluorescence is diminished when compared with that seen in Fig. 5 (x 250).

animals which develop an immediate nephritis, with or without the nephrotic syndrome, appear to have bound more of the antiserum in the glomeruli than have animals injected with antisera which produce nephritis only after a latent period. By the same method it is found that in an animal injected with nephrotoxic antiserum the glomeruli retain the foreign protein for months. It is possible that this immunologic assay of bound antibody will be useful in establishing a relation between localized antibody and renal disease.

CHAIRMAN HEYMANN: Thank you, Dr. Seegal. We will postpone the discussion until we have heard Dr. Lange, who will now discuss "Delayed nephritis due to avian antiserum."

B. Delayed Nephritis Due to Avian Antiserum

DR. KURT LANGE (New York Medical College): Ladies and Gentlemen! We have very little to add to the beautiful studies by Dr. Seegal except possibly to one point. namely, the statement that the delayed and immediate nephritis are the same, to which we may have to take exception.

Our work has extended over the past eight years, with a great number of collaborators, especially Mr. Wenk and recently, Dr. Noble. But a great many others have collaborated in them with much merit.

One further credit should be given; this is to a technique which was described by Dr. Carl Cohen, of the Jackson Memorial Laboratory in Bar Harbor, which I think I will show to you very shortly. This is a technique for drawing blood from a rabbit.

Dr. Carl Cohen[10] has described how one can easily take blood from the central-ear artery of a rabbit. You transilluminate the ear and introduce a fine 24-gauge needle attached to an adapter into the central ear artery. When the first drop of blood issues, an evacuated 20 ml. test tube with diaphragm stopper is connected to the other needle point of the adapter. Thus, one can get blood samples as often as one wants, very quickly and sterile if one so desires. We have taken up to 8 samples per day for 5 consecutive days, from the same animal, without any difficulty. That has helped a great deal in carrying out the different immunological and biochemical tests which we have had to perform on these animals.

Experimental nephritis, as Dr. Seegal has stressed, is important not only as an interesting pathological entity, but also for the understanding of human glomerulonephritis and nephrosis and, if it is similar, as a proving ground for therapeutic approaches.

It is, therefore, necessary that the experimental disease simulate as closely as possible the human disease. In glomerulonephritis, for example, there should be a time interval between the streptococcal sore throat and the onset of the disease, which may vary between 5 and 15 days; proteinuria, and in most instances hematuria, except in the nephrotic stage, and a fall in serum complement for prolonged periods of time in

[10] Cohen, C., A Method for Bleeding Rabbits. Am. J. Clin. Path. 25: 604, 1955.

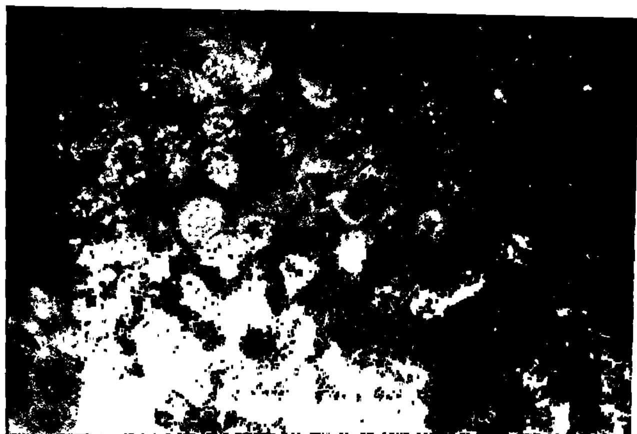


Fig. 7. Section of kidney from rat SDB963, see Fig. 5, first treated with undiluted and untagged duck anti-rabbit-globulin, then with fluorescein-tagged duck anti-rabbit-globulin. Viewed under untraviolet light the specific glomerular fluorescence is absent (x 250).

most instances; finally, a subacute phase can occur after the acute phase, uremia can occur in the beginning and at the end of the disease, and complete healing may be observed.

In our opinion, there are two entirely different types of disease, which we would like to divide and call the monophasic and biphasic disease.

The monophasic disease is the one which Dr. Seegal has beautifully described to you, where kidney emulsion of a rat is injected into a rabbit in numerous immunizing injections. Finally, blood of the rabbit is drawn and the serum is injected intravenously into the rat. Without any time delay, if the serum is potent enough, there is appearance of disease, certainly within less than 24 hours. As many observers (Dr. Pfeiffer[11], who is with us here today; Dr. Heymann[12], and we[13]) have described, complement falls very sharply for less than 24 hours; then it returns rapidly to normal. A direct antigen-antibody reaction between the glomerulus and the antiglomerular mammalian antibody has taken place, using complement in the reaction.

The same thing can be shown in isolated kidneys with perfusion experiments, as we have described[14]. When an isolated rat kidney is perfused with rat blood which has a certain complement content, and then anti-rat-kidney rabbit serum (so-called nephrotoxic serum) is added to this blood. Within one passage through the kidney, all or most of the complement is removed, even if the ureters are ligated, so that there is no possibility that the complement could have escaped into the urine.

The biphasic disease, in contrast, appears to be entirely different. The important feature appeared to be the time delay which occurs with great regularity and, we found, without exception between the injection of the antiserum and the appearance of the disease.

We have tabulated the first individuals to describe a given experimental arrangement producing either mono- or biphasic disease (Table 3). In 1900, Lindemann first described that he could produce nephritis in the rabbit by giving guinea pig anti-rabbit-kidney sera. The recipient animal was a rabbit; and there was no time delay between injection of the nephrotoxic serum and the appearance of the disease. Others who described a monophasic nephritis were: In 1933, Masugi described rat-rabbit-rat disease, without a delay; Smadel: rat, rabbit, rat; Dr. Seegal, in 1946, rat placenta, rabbit, rat; Stabley, dog, rabbit, dog, no time delay. Recently, Mellor's rabbit, goat, rabbit, no time delay. In all of these there was always immediate disease.

[11] Pfeiffer, E. F., Schoffling, K. and Bruch, H. E., Serumkomplement und Masugi-Nephritis der Ratte. Verhdlg. d. Dtsch. Ges. f. inn. Med. 59. Kongress, 1953.

[12] Stavitsky, A. B., Hackel, D. B. and Heymann, W., Reduction of Serum Complement Following in vivo Tissue Antigen-Antibody Reactions. Proc. Soc. Exper. Biol. & Med. 85: 593-596, 1954.

[13] Lange, K., Complement Components in Human Nephritis and Nephrosis. Proc. Sixth Ann. Conf. Nephr. Syndr. 77, 1954.

[14] Lange, K. and Wenk, E. J., Investigations into the site of Complement Loss in Experimental Glomerulonephritis. Am. J. Med. Sc. 228. 454-460, 1954.

TABLE 3

PRODUCTION OF EXPERIMENTAL GLOMERULONEPHRITIS

Author	Year of Publication	Donor Organ	Intermediary Antibody Producer	Recipient Animal	Period of Delay
Lindeman	1900	rabbit kidney	guinea pig	rabbit	none
Masugi	1933	rat kidney	rabbit	rat	none
Smadel	1937	rat kidney	rabbit	rat	none
Seegal	1946	rat placenta	rabbit	rat	none
Heymann	1951	rat kidney	rabbit	rat	none
Stabley	1956	dog kidney	rabbit	dog	none
Stickler	1956	dog kidney	rabbit	dog	none
Mellors	1956	rabbit kidney	goat	rabbit	none
Masugi	1934	rabbit kidney	duck	rabbit	more than 5 days
Fouts	1941	dog kidney	chicken	dog	more than 5 days
Kay	1942	rabbit kidney	duck	rabbit	more than 4 days
Lange	1951	rabbit kidney	duck	rabbit	more than 4 days
Lange	1955	rat kidney	duck	rat	more than 3 days
Stavitsky	1956	rat kidney	duck	rat	more than 3 days-immediate in 10%
Lange	1957	rabbit kidney	chicken	rabbit	more than 4 days

Then, in 1934, Dr. Masugi described another type of disease, without being very much aware of the difference between the two observations. He injected rabbit kidney emulsion into ducks, and the rabbit was then the recipient animal for the duck immune serum; there was a time delay of at least 5 days between injection of the immune serum and the appearance of clinical disease.

Fouts and Corcoran and Page have injected dog kidney into chicken and the resultant immune serum into dogs. There was 4 days delay. In our own studies, which we presented before this meeting in 1951, we used a rabbit-duck-rabbit system: with 4 days delay before the disease became noted. Stavitsky has taken rat kidney, injected it into ducks, then the duck serum into rats. He has found more than 3 days delay except in about 10 per cent of the animals, where an immediate proteinuria was noted.

We have taken recently rabbit kidney, given it to chickens and then injected the chicken immune serum back into the rabbit, and again a time delay of more than 4 days occurred. The same applies in our observations on rat kidney given to ducks and the serum injected back into the rat.

Dr. Seegal's group has recently published a paper which we purposely did not put on the table because we did not know where to put it. In their paper, they stated that they have produced, with highly potent duck sera, immediate disease in the rat.

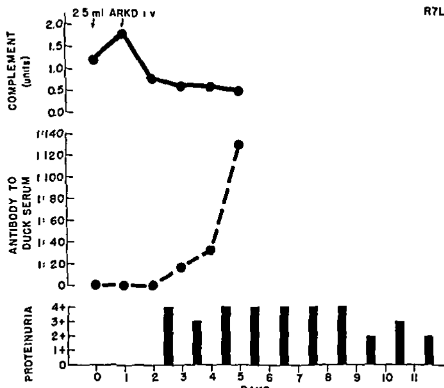


Fig. 8. An example of a "biphasic" type of heteronephrotoxic serum renal disease in the rat. Note that proteinuria occurs 2-3 days after first injection of anti-rat kidney duck serum. As antibody titers rise, serum complement levels fall.

If one analyzes the published charts of Hasson, Bevans and Seegal[15], one will notice that 3 injections of immune duck serum are given 24 hours apart, as described in the text, the so-called "immediate disease" occurring with the most potent sera 24 hours after the last injection.

In our opinion, counting of days of delay should start on the first day of immunization; then one would get not an immediate disease but a 4 days delay in the appearance of the disease with the highly potent sera that Dr. Seegal's group had. We would like to group these experiments too, into the delayed disease category, unless Dr. Seegal can tell us otherwise.

We have, then, a basic difference. In the first group, all antibody producers are mammals and their immune sera produce immediate disease; and, in the second group, all antibody producers were avians, giving always (with very few exceptions) delayed disease. We have tried, with all means we knew of, to produce a duck serum against rabbit kidney which gives immediate disease. We have not succeeded in doing so.

The rat, however, has a peculiarly bad reputation as a poor antibody producer, a reputation which it apparently does not deserve.

When 6 rats were given anti-rat kidney duck serum in very large amounts (2.5 ml.) we noticed that within 3 days, antibodies against duck serum appeared in the rat. In 7 days, all 12 animals of this group *had very high antibody titers against duck serum.*

We have other data to show that within 3 days in other groups, with other batches of duck immune serum, antibodies can be found in the serum of the recipient rats, and the disease occurs between the third and fourth day in these animals. Apparently, the rat is not such a poor antibody-former.

Figure 8 shows an example of such a rat, to whom anti-rat-kidney duck sera was given. Between the second and third day after the first injection, proteinuria occurs. At the same time, the antibodies to duck serum rise very rapidly. Serum complement falls to below normal levels simultaneously with the rise of the antibody.

Kay has given this biphasic disease a very interesting interpretation. It had apparently escaped *Masugi's attention* that there was a basic difference between the monophasic and the biphasic disease.

Figure 9 represents Kay's[16] idea about the mechanism of the disease. This is best described as follows:

One gives rabbit kidney localizing duck serum to a rabbit. This serum forms a coat on the glomerulus of the recipient rabbit. The coat consists of duck serum, which has a special affinity to the glomerulus. At the same time, the duck serum also gets into

[15] Hasson, M. W., Bevans, M., and Seegal, B. C., Immediate or delayed nephritis in rats produced by duck anti-rat kidney sera. Archives of Pathology 64: 192-304, 1957.

[16] Kay, C. F., The Mechanism by which Experimental Nephritis is Produced in Rabbits Injected with *Nephrotoxic Duck Serum*. J. Exper. Med. 72: 559-571, 1940.

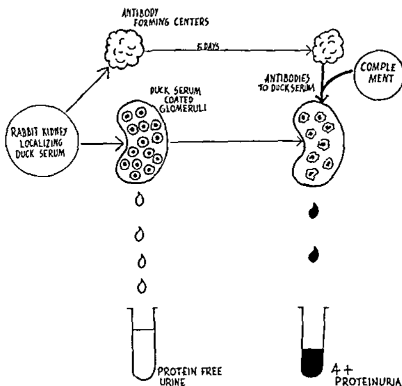


Fig. 9. Mechanism of heteronephrotoxic serum nephritis, according to Kay (1940) [16] The glomerulus is coated with anti-rabbit kidney duck serum. Systemic antibodies against this serum, formed over a 4-6 day period, localize on and react with the "coated" glomerulus, causing acute nephritis.

the general circulation. Antibodies are formed to the duck serum, and *when they appear* within 4 to 6 days, these antibodies react with the duck serum on the *glomerulus* and acute glomerulonephritis results, with a fall in complement. The behavior of complement is our addition, which was not described in Kay's original scheme.

At the onset of disease, antibodies against duck serum appear in the rabbit's blood. This hypothesis was supported by the fact that when antibody formation, as Kay has shown, was suppressed by massive total body X-ray radiation, the disease would not occur, and antibodies to duck serum did not appear in the bloodstream; thus, the occurrence of the disease was suppressed. Everybody, including ourselves, believed fully in this explanation.

In general, our immune sera, unless we had a special purpose in making them especially strong, were produced by giving ducks 10 cc. of a 10 per cent rabbit kidney emulsion of well-flushed rabbit kidneys every second day for two weeks, then one week's rest, then again injections every other day for two weeks; then, again, one week's rest, then blood was taken by heart puncture.

Each pool of duck sera consisted of the blood of at least 7 but mostly 10 ducks, or when we used chickens, 10 chickens in order to get uniformity of results, not relying upon one duck or chicken alone.

The usual dose of immune serum given to a rabbit was 1 cc. per lb. of body weight on 2 successive days, or the gamma globulin of this amount of immune serum which produces a disease just as well as the total serum, omitting the other antigenic proteins.

We have tried to produce, as I stated before, a more potent serum to avoid the delayed onset time interval if possible, but we have not succeeded. In 263 rabbits in whom we have produced the disease, we have never had one which had the disease before the fourth day. We could produce an immediate severe hematuria and proteinuria when we gave 15 cc. of the serum in one injection. With this amount, approximately 60 per cent of the animals died within 24 hours, but, on autopsy, it could be shown that these animals had petechiae throughout all mucous membranes of the body. We apparently had an immediate serum reaction which had nothing to do with an acute glomerulonephritis, for in the 40 per cent which survived the hematuria, proteinuria disappeared within 2 or 3 days and the rabbits after 5 to 6 days developed full-blown nephritis, of which we are going to see several examples.

In the first instance, there was no fall in complement. Those which recovered, and then got real disease 2 or 3 days later, showed an abrupt fall in complement and the appearance of antibodies against duck serum in the second phase.

What happens clinically to a rabbit which receives anti-rabbit-kidney duck serum? Figure 10 shows a typical example; two injections of 1 ml/lb. of anti-rabbit-kidney duck serum are given on days 0 and 1. There is no proteinuria until the fifth day; then proteinuria occurs. The next day, the animal becomes anuric. Complement simultaneously falls; and, with the appearance of anuria, antibodies (precipitins) to the duck serum appear in the blood. With the onset of disease the BUN rises.

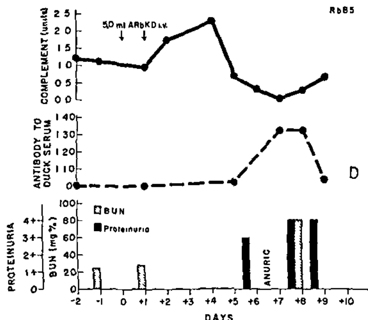


Fig 10. Clinical course of heteronephrotoxic serum nephritis in a rabbit injected with anti-rabbit kidney duck serum. Proteinuria, anuria, and azotemia occur 5 days or more after injection.

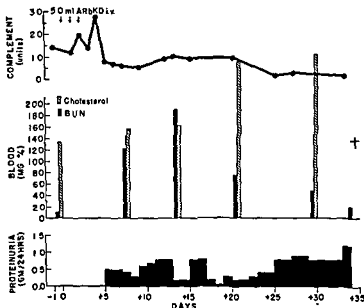


Fig. 11. Increased severity of induced nephritis in the rabbit, following injection of anti-kidney serum for 3 successive days.

Figure 11 shows an animal to which a larger dose of the same serum was given, by giving it for 3 successive days; the proteinuria is more severe, cholesterol rises, complement falls, and the BUN rises very dramatically to values of 120, 160, then decreases. At the end, after 35 days, the animal was sacrificed.

I wish to stress one thing which Dr. Seegal has already mentioned, that you can produce with less potent sera or with smaller doses of sera, a delay longer than 5 days; but you cannot shorten this interval to less than 4 days in our experience. You can delay the clinical and histologic appearance of the disease to the seventh, eighth and ninth day by using smaller amounts of the serum or by diluting it.

TABLE 4

MALE ALBINO RABBIT, 3390 gm.

Day of exper.	Procedure	Complement units	URINE Volume	Prot. g. /24 hrs.	RBC HPF	Casts	BLOOD BUN	Cholest.
-1		1.4	90	--	--	--	12	134
0	Anti-rabbit-kidney duck serum 5 cc. i.v.		110	--	--	--		
1	"	1.2	40	--	--	--		
2	"	1.9	20	--	--	--		
3		1.4	45	--	--	--		
4		2.7	60	--	--	--		
5		0.77	15	0.5	15	occ.		
6		0.66	45	0.5	15	--		
7		0.58	40	0.43	30	--	122	178
8			33	0.3	50	--		
9		0.52	35	0.6	40	many		
10			18	0.7	30	many		
11			35	0.8	45	many		
12		0.93						
13		0.99	100	0.2	20	few	190	162
15		0.87	60	0,8	25	occ.		
16			25	0.8	10	--		
17			60	0.2	--	--		
18			15	0.05	--	--		
19			80	0.3	occ.	--		
20		0.92	110	0.2	7	--	76	273
21			130	0,2	7	--		
22			170	0.3	9	--		
23			90	0.4	15	--		
25		0.14	140	0.8	occ.	--		
27		0.51	180	0.9	6	--		
29			250	0.8	20	--	49	283
33		0.13	210	1.2	--	--	20	
34	Found dead, autopsy performed.							

Fig 12. Effect of dilution of anti-rabbit kidney duck serum on occurrence of renal disease (proteinuria). Note that onset of proteinuria could be delayed 4-5 days by diluting same potent serum.

Table 4 represents the course of one animal followed for 35 days, which then died. Around the fifth to seventh day together with the appearance of proteinuria, marked oliguria appears. Red cells appear in the urine around the seventh to tenth day, but later on disappear completely. The BUN first rises sharply then comes down slowly, cholesterol rises quite markedly, and complement falls to very low values, at the same time the disease begins, from 2.77 to 0.77, from one day to the next.

Up to this point, everything appeared in full agreement with the concept as suggested by Kay.

But the next experiments did not bear that out. Since Kay had stated that it is the antibody against duck serum which produces the disease, by forming a union with the duck serum which had coated the glomerulus, we thought we might need very little of the localizing anti-rabbit-kidney duck serum to achieve the coating and we could replace the rest with duck serum; the animal would form antibodies to that duck serum just as well, and we would not have to go through the long procedure of producing so much anti-rabbit-kidney duck serum.

Figure 12 shows on the bottom a normal experiment with the same nephrotoxic serum. The animal received 7.5 ml. anti-rabbit-kidney duck serum on two successive days. On the fourth day, proteinuria set in, discovered the first time on the morning of the fifth day. The antibodies against duck serum appeared in the blood, then rapidly rose to a high titer.

We then took only 2.5 ml. of anti-rabbit-kidney duck serum and added 5.0 ml. of plain duck serum and did the same on day +1.

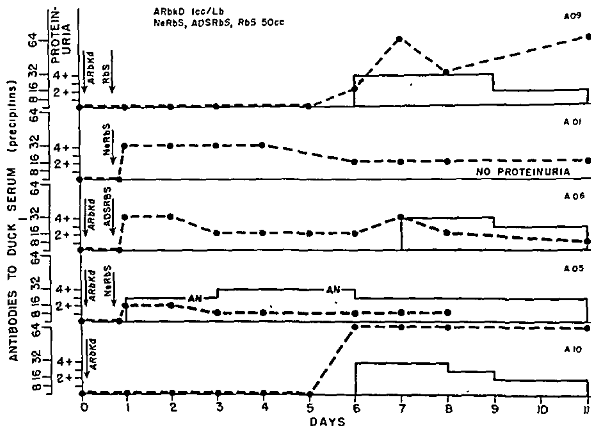
You will note that again on the fourth to fifth day, the anti-duck serum titer rises very markedly; but no disease occurred. Disease was only discovered on the morning of the seventh day; that meant proteinuria started between the sixth and seventh days.

This was a very disquieting finding, namely, that the titer against duck serum apparently was not too closely related to the occurrence of the disease.

Then we took 1.5 ml. of anti-rabbit-kidney duck serum, and added 6.0 ml. normal duck serum. We got a nice rise of titer against duck serum, but still more delay in the occurrence of the disease. Between the eighth and ninth day proteinuria appeared and with it the other clinical features.

We then wanted to know whether the duck serum added anything to the clinical state of these animals. We therefore added normal human serum instead of normal duck serum as a filler; i.e.: we gave 1.5 ml. anti-rabbit-kidney duck serum and 6.0 ml. of human serum. You see in the upper part of Figure 12 that the duck serum titers rise very little; but the disease occurs just the same as it did before with 0.5 ml. of anti-rabbit-kidney duck serum on the eighth to ninth day.

This suggested to us two possibilities: one possibility was that the antibody to the duck serum was not primarily responsible for the disease, but that another antibody which is produced simultaneously must be responsible for the disease. Or a second possibility was that the "glue", the coating, was not sufficient for the glomerulus to attract enough of the anti-duck serum antibodies.



ARbKD=antirabbit kidney duck serum, ADSRbS=antiduck serum rabbit serum, NeRbS=nephritic rabbit serum, RbS=rabbit serum

Fig. 13. Induction of "immediate" disease without latent period by transfusion of serum from nephritic rabbit into another rabbit injected 1 day previously with anti-rabbit kidney duck serum. A demonstration of fixation of preformed antibodies on a sensitized ("coated") glomerulus. Row 1 - control. Anti-rabbit kidney duck serum injection followed [illegible] followed. Row 2 [illegible] occurred. Row 3 [illegible] serum does not al[illegible] course of anti-rabbit kidney duck serum disease. Row 4 - shows immediate onset of disease in sensitized rabbit following transfusion of nephritic rabbit serum.

Against the latter possibility was the experiment with human serum where the disease occurred just as if we had given only 1.5 cc. anti-rabbit-kidney serum.

With the next group of experiments (Fig. 13) *we found suddenly that we could produce* immediate disease, with a certain experimental setup.

The animal in the bottom section of the chart (A10) was given 1 cc. per lb. of anti-rabbit-kidney duck serum, one injection only.

This animal showed proteinuria on the sixth to seventh day; on the sixth day, the antibodies to duck serum rose; and one could still establish a good relationship.

Then we did the following: We took rabbits who had severe nephritis for 2 or 3 days due to duck *immune serum given 7-8 days before, bled them* and transfused their serum as shown in Row 2 - to normal rabbits.

When this was done, the normal rabbits did not get any disease. The blood of the nephritic rabbits which had high antibody titers to duck serum did not produce disease in a healthy animal.

If, however, we transfused the same amount (50 ml.) of nephritis serum to a rabbit which had one day before received anti-rabbit-kidney duck serum, we could produce the disease immediately. Within 24 hours, the animal showed severe proteinuria and the *delay had completely disappeared (Row 4). Such an animal does not have to produce* antibodies any more; it obtains the antibodies transferred by the transfusion from the nephritic animal onto a kidney which is sensitized.

To disprove the point that the duck serum itself is the culprit in this situation, we sensitized rabbits to plain duck serum; not to anti-rabbit-kidney duck serum, but to plain duck serum. We then transfused their blood, after previous sensitization of the recipient animal with anti-rabbit-kidney duck serum. We obtained a disease in the recipient animal which was in no way faster than when we had given the anti-rabbit-kidney duck serum alone, i.e., the delay was present again and was not abolished by the transfused anti-duck serum antibodies (Row 3).

We have had recently - in the last series we have studied - three exceptions where animals with this latter type of disease also got a rather rapid occurrence of proteinuria with a short interval instead of the delayed type which they would get with the injection of nephrotoxic serum alone. We do not have an explanation for these rare exceptions as yet. Complements did not fall; and they did not get a rise in BUN, as the animals with the transfusion nephritis showed promptly.

Finally, in the top row a control is shown. Anti-rabbit-kidney duck serum was given, followed by plain rabbit serum; and, again, the disease occurs as if only anti-rabbit-kidney duck serum were given, with the usual delay.

In short, these experiments seem to show that we transfer in the blood of nephritic rabbits, an antibody to the localizing factor, not to duck serum which is either a modified duck serum, a duck serum with a special hapten, or a *gamma globulin* of its own without duck serum specificity.

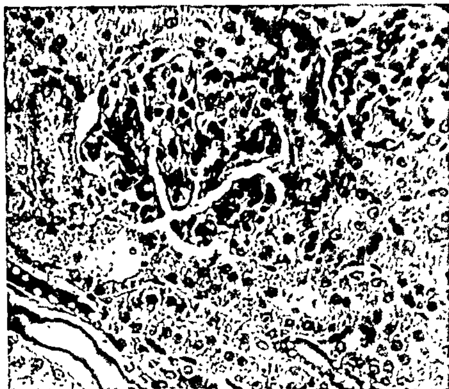


Fig. 14. Glomerular proliferative and exudative reaction in "immediate" disease, 24 hours after transfusion of nephritic rabbit serum to previously sensitized rabbit. (H & E stain.)

CHAIRMAN HEYMANN: May ask you on which day you obtained the serum?

DR. LANGE: This serum which was transfused was usually obtained two or three days after the appearance of the disease; that means on the sixth or seventh day after injection of the nephrotoxic duck serum.

DR. GOODMAN: One more question, Dr. Lange. When you made the antibody against duck serum, was it an antibody to normal duck serum?

DR. LANGE: They were given plain, not nephrotoxic duck serum instead of anti-rabbit-duck serum. They formed antibodies to duck serum, as you saw in the last slide; but this disease is not transferable, or does not contribute to immediate apppearance of the disease.

Figure 14 is a histologic section of the kidney of one animal which Dr. M. Wachstein, who performs the histologic and histochemical studies on our material, was kind enough to supply. One day after the transfusion of nephritic serum into a sensitized animal, i.e., one day after the animal was transfused and showed the immediate disease, this animal was sacrificed. You see the hematoxylin eosin stain 24 hours after the transfusion and 48 hours after the anti-rabbit-kidney duck serum injection. Where all our other animals which were not transfused with nephritic rabbit serum - and Dr. Wachstein has done about 150 such specimens - show nothing in the kidney sections, this animal showed large amounts of proteinaceous material in the tubuli. There were proliferative changes in the glomeruli, as evidenced by a marked increase in cellularity, and exudative changes indicated by proteinaceous material in and between the glomerular tufts.

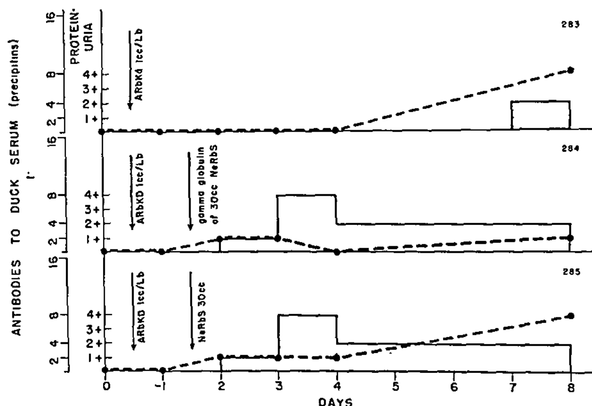
Figure 15 shows one more point; the transfusion of nephritis-producing serum into a sensitized animal can be done with the gamma globulin of the blood of the nephritic rabbit alone; you do not have to give the entire serum.

In the bottom row is an animal which received anti-rabbit-kidney duck serum, 1 ml. per lb., on day zero, and received 30 ml. of nephritic rabbit serum on the next day; that means, serum of a rabbit which had the full-blown disease for two days. Within a day, the recipient animal comes down with an acute glomerulonephritis of the type you have just seen.

When we used only the gamma globulin from the 30 ml. of that same nephritic rabbit serum and injected it into another sensitized rabbit, we see almost an identical picture (Row 2). The disease is transferable by the gamma globulin alone, and does not require the total serum, making it more probable that this is a true antibody.

Finally, in the top row is shown a control where only 1 ml. of anti-rabbit-kidney duck serum per lb. was given. It is evident that the lower two rows indicate a transfusion disease in a sensitized animal.

Figure 16 shows the difference in the concept which Kay had proposed and which we propose for this disease. Kay suggested that the rabbit kidney localizing serum localizes duck serum on the glomerulus of the recipient animal, that antibodies to duck



ARbKD = antirabbit kidney duck serum, NeRbS = nephritic rabbit serum

Fig. 15. Row 1 - [illegible] following [illegible] of 1 ml. of [illegible] kidney duck serum. Row 2 - [illegible] rabbit serum will [illegible] the antibody is carried in the gamma globulin fraction. Row 3 - illustrates "immedeate" disease following transfusion of nephritic rabbit serum into sensitized rabbit.

serum are formed; and that, when these antibodies appear in the circulation, the previously coated glomerulus becomes damaged or destroyed *with the* use of large amounts of complement and proteinuria occurs.

From our experiments we feel fairly convinced that the following is a better theory: *when one gives rabbit kidney localizing duck* serum, a localizing factor which is not duck serum, and possibly does not have the characteristics of duck serum, but is a gamma globulin, coats the glomeruli. Antibodies are formed to this localizing factor. When these antibodies to the localizing factor appear in the circulation, the disease occurs with the use of large amounts of complement.

Therefore if this were in any way transferable to the human, one could understand some experiments which were reported from Italy some time ago, in which the transfusion of sera of nephritic individuals to normals, did not lead to any kidney damage in the normal. If this holds true at all, the only reasonable experiment would be to take nephritic sera, if possible of the same individual, drawn while he has an acute episode of nephritis or nephrosis, and inject this serum after he has had a remission. Then we are dealing with a sensitized kidney and the individual should perhaps come down with a short-lasting relapse until this antibody is disposed of.

Such studies are under way at present; I do not know whether they will show the important bridge between experimental animal and human disease.

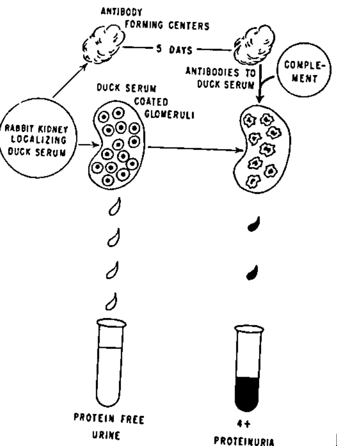
The next and last group of experiments was concerned with cortisone suppression of the experimental disease.

If this is a disease in which the antibody formation of the recipient animal plays a decisive role, as Kay has suggested and as we wish to modify, cortisone if given in sufficient amounts should be able to suppress antibody formation and thus the disease.

Table 5 shows three animals given 5 cc. of anti-rabbit-kidney duck serum on day zero and day 1. On day 5 or 6 the rabbits came down with a 3+ or 4+ proteinuria. The BUN rises quickly; this represents the normal course of this disease.

The same amount of anti-rabbit-kidney duck serum was given to animals which, at the same time, *received 60, 40 or 35 mg. of cortisone per kg. of body weight per* day. With this medication the onset of disease was much delayed and the disease was much milder, there was hardly any rise in BUN, and much less proteinuria and much lower antibody titers than in the controls. We have studied this modification of the disease in about eighty animals. We found that the minimal amount of cortisone which will induce a significant suppression of this disease is 5 mg. per kg.

The best results, without mortality due to cortisone or significant electrolyte disturbances, but with good depression of antibody formation and improvement of disease, was 20 mg. cortisone per kg. per day. With that dose, a very marked or complete suppression of the disease occurrred, indicating that if one interferes markedly with antibody formation, this disease can be altered decisively.



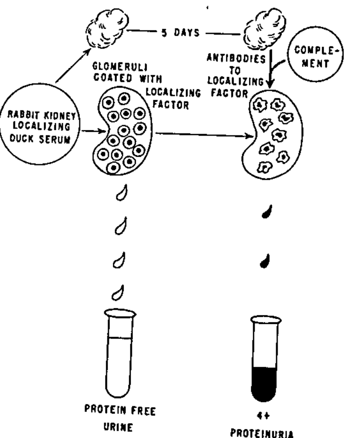


Fig. 16. Modification of Kay's theory. coating of rabbit glomeruli with duck anti-rabbit kidney gamma globulin. Rabbit antibodies against this localizing factor are formed, combine with complement and produce disease.

TABLE 5

EFFECT OF CORTISONE ON ANTI-RABBIT-KIDNEY DUCK SERUM NEPHRITIS

RABBIT		DAYS -2	0	+1	+5	+6	+7	+8	+9	+10	+11	+12	+13	+14	+15	+33
B 5*	Therapy		NONE													
	Urine Prot.	-	-	-	3+	AN	4+	4+	4+	4+	D					
	BUN mg%	14		12				80								
B 6	Therapy		NONE													
	Urine Prot.	-	-	-	-	3+	1+	2+	4+	3+	3+	1+	2+	2+	2+	
	BUN mg%	11			12			53								
C 5	Therapy		NONE													
	Urine Prot.	-	-	-	-	4+	4+	AN	4+	1+	3+	2+	2+	2+	2+	1+
	BUN mg%	17			15				52						53	32
F 1	Therapy	← 60 mg Cort. →														
	Urine Prot.	-	-	-	AN	-	-	TR	1+	2+	1+	2+	2+	2+	2+	
	BUN mg%	15									25					
E 4	Therapy		← 40 mg Cort. →													
	Urine Prot.	-	-	-	-	-	-	2+	2+	2+	2+	2+	2+	2+	3+	
	BUN mg%	16							20						14	
E 8	Therapy		← 40 mg Cort. →													
	Urine Prot.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1+
	BUN mg%	12								21					14	18
C 11	Therapy		← 35 mg Cort. →													
	Urine Prot.	-	-	-	-	-	TR	2+	2+	2+	3+	3+	3+	2+	1+	-
	BUN mg%	18							13						21	9
D 1	Therapy		← 35 mg Cort. →													
	Urine Prot.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	BUN mg%	19			21				15						22	10
D 2	Therapy		← 35 mg Cort. →													
	Urine Prot.	-	-	-	-	-		1+	1+	2+	3+	3+	3+	3+	1+	-
	BUN mg%	16			23					21					18	4

All animals received 5 ml. anti-rabbit-kidney duck serum i.v. on days 0 and 1+.

*16 additional control animals either died early or showed evidence of severe proteinuria starting the fifth day and uremia.

AN = anuria TR = trace D = died

CHAIRMAN HEYMANN: We have heard two very stimulating presentations and I would like to open both of them for discussion.

DR. DAVID GITLIN (Children's Hospital, Boston, Massachusetts): Dr. Heymann, I would like to suggest a unifying factor between Dr. Lange's experiments and those of Dr. Kay.

When an antibody combines with an antigen, it is no longer the original gamma globulin which it was to begin with. The structure opens up; and SH groups are now exposed. It is entirely conceivable that an antibody would be formed against this denatured gamma globulin, which would react not only against the denatured gamma globulin (now an antibody in reaction), but against normal gamma globulin as well. I think this would explain all of the results which have been presented.

I would like to ask Dr. Lange whether he has conceived of the idea of reacting glomeruli with duck antiserum, washing these, injecting them into an animal which would not get nephritis. The glomeruli should contain the potentiating factor he is looking for.

DR. LANGE: As an emulsion?

DR. GITLIN: As an emulsion. These rabbits should not get nephritis; they should develop antibodies against whatever material is now on the glomeruli, derived from duck serum. Such an antibody would potentiate the kidney disease to give the immediate type of response you have seen with your antiserum obtained from rabbits with nephritis. It might even be reduced to a more simple concept by taking a specific precipitate, using some heterologous material with a duck antiserum, and then using specific duck precipitate as the immunizing factor. Would this not have the potentiating factor you are looking for?

I do not think Dr. Kay ever suggested that it was the whole serum which was causing the specific antibody response in the rabbit; it was taken for granted that this was a localizing antibody, and it was a rabbit antibody to the duck antibody which was giving the reaction.

DR. LANGE: What we are doing at present is to elute it from such an absorption, not to give the entire emulsion, but to elute it with salicylate or --

DR. GOODMAN: Along the same line of reasoning, if you immunize a rabbit not with normal duck serum, but with nephrotoxic duck serum, could you make an antibody against the specific antibody globulin you are interested in? Dr. Baxter and I have discussed the possibility that the antibody against a normal duck globulin may not react with what may be an altered antibody globulin? Have you tried that?

DR. LANGE: No; that is a good idea.

DR. FREDERICK G. GERMUTH, Jr. (Department of Pathology, Johns Hopkins Medical School): I would like to ask Dr. Stone if anyone has been able immunologically to differentiate one type from another by making antibodies to an antibody. Dr. Grabar has been trying to do that.

DR. GITLIN: I would like to emphasize that this is not quite the same. You are trying to distinguish different gamma globulins as different antibodies. This is not the same as distinguishing a normal gamma globulin from an antibody reaction.

DR. STONE: Frank Adler has injected immune precipitates from rabbit-anti-guinea-pig serum, plus guinea pig serum, back into guinea pigs. There is no indication that the rabbit antibody which is in this complex has been altered in any way. The antibodies formed by the guinea pig against rabbit antibodies, whether free in serum or whether on a complex, are identical.

DR. GITLIN: You mean he has two types of gamma globulin in guinea pigs, one formed from a specific precipitate, and the other the isolated, undenatured gamma globulin?

DR. STONE: That is right.

DR. GOODMAN: How about normal gamma globulin? Is that present, too?

DR. STONE: On the question of normal gamma globulin, Grabar, of course, says that all globulins are antibodies; and, of course, all antibodies are globulins.

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DR. GERMUTH: And got antibodies to that, then put them back into that animal.

DR. LANGE: We did not get an accelerated response.

DR. MILTON RAPOPORT (Children's Hospital of Philadelphia): Are there other antibodies in the sera which you prepared which are nephrotoxic?

DR. LANGE: In all probability there are, there are numerous proteins.

DR. RAPOPORT: The reason I ask this is that, many years ago, in giving a child repeated injections of liver extract, we found that they got a severe local reaction with the liver extract which had a high titer of Forssman's antibody; and then, using liver extract prepared from a species which also had Forssman's antigen - I think one such was beef - we did not get any reaction. Presumably, there must have been Forssman's antigen in most of this liver extract.

Is there a difference in the capacity of the duck or the rabbit to form antibodies? The rat kidney has Forssman's antigen; but there are a lot of species., You are just focused on the nephrotoxic serum.

Where this fits in, I do not know; but you have either an accelerator or inhibitor of this accelerated or delayed reaction, depending on whether you are using rabbit or duck antiserum. If I have the time sequences right, with the rabbit sera you get an immediate nephritis. This means there are prosthetic groups in it which would damage the kidney. The duck ought to produce a similar serum which fits the kidney and does not damage the kidney after five days.

DR. LANGE: It is very simple why it does not. An avian antibody cannot fix mammalian complement. Thereby, you get antigen-antibody union; but you do not get a complement-binding, tissue-destroying reactor. Only when a mammalian antibody (namely, the one which is formed by the rabbit itself) combines with a mammalian antigen does the complement-binding tissue destroying the antibody occur.

Dr. Heymann and Dr. Pressman have shown very beautifully that, in the duck, there is an immediate union. The complement does not fall; and nothing happens to the glomerulus.

DR. RAPOPORT: If you remove the complement from a rabbit, do you get a rabbit nephrotoxic serum; would you get nephritis?

DR. LANGE: That is what we are trying. Dr. Osler has shown that, when you give preparations which destroy the complement, and thus make complement completely inactive, a normal-antibody skin reaction does not occur.

CHAIRMAN HEYMANN: I believe that things are a little complicated, by virtue of the fact that we are talking about duck serum which has been produced by different schedules of injections. I believe, for instance, that Dr. Seegal has much more potent sera than we have. Furthermore, it is confusing to have anti-kidney sera obtained from ducks against rats, and to talk simultaneously about anti-kidney sera obtained from ducks against rabbits. That also may be a different story.

We have for many years adhered to the view that duck anti-kidney sera produce latent periods in rats, while anti-kidney sera obtained from rabbits do not.

A few years ago we investigated the length of the latent period in relation to the intensity of the disease observed in seventy rats injected with anti-rat kidney serum obtained from ducks[17]. The latent period in the mild diseases varied from one to thirty days. In moderately severe disease, the latent period varied from four to twenty days. With very potent sera and resulting severe disease we had, like Dr. Seegal and unlike Dr. Lange, marked proteinuria within the first eighteen hours after the serum injection.

DR. LANGE. What happened the next day?

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[17] Stavitsky, A. B., Heymann, W. and Hackel, D. B., Relation of Complement Fixation to Renal Disease in Rats Injected with Duck Anti-kidney Serum, J. Lab. & Clin. Med., 47: 349, 1956.

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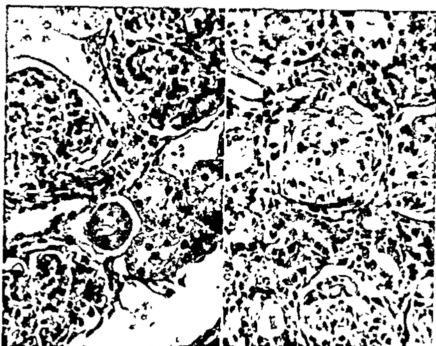


Fig. 17. Difference in reaction of rat kidney following injection of potent (on right) or weak anti-rat kidney rabbit serum. Animal on left had delayed onset of disease (7th vs. 3rd day), little proteinuria, and a proliferative rather than regressive lesion.

DR. LANGE: We have had that too; but, the day after injection they were free of disease, which then appeared in three, four, or five days.

CHAIRMAN HEYMANN: We have not seen that. We believe that very potent duck sera may produce immediate disease, without detectable latent period. The question that Dr. Seegal has raised has to be answered, whether or not the latent period noted after the use of avian anti-kidney sera is only dependent on the potency of the sera used. This I do not believe. It remains true that in moderately severe disease produced in rats by duck sera there is a latent period often of considerable length, whereas in moderately severe disease in rats produced by rabbit sera a latent period is not noted.

If one injects rats with not very potent anti-kidney sera obtained from rabbits, slight to moderate degrees of proteinuria may be noted after only two to four days. This, however, does not necessarily mean that one is dealing with a true latent period. It may be due to the fact that a slowly increasing proteinuria does not reach abnormal degrees for four days, even though it may be increasing day by day. It could very well be that electronmicroscopy would show definite changes of the foot processes in this early phase, when proteinuria is not yet detectable and when light microscopy would fail to show histological alterations.

In addition to these points, we have to contend with the fact that a rat injected with anti-rat-kidney serum obtained from rabbits has an immediate complement drop, whereas a rat injected with anti-kidney serum obtained from ducks has a complement drop only after four, five or more days. At this time, the latter produces anti-duck serum protein antibodies which were not detectable before. Neither the hypothesis of Lange, nor the older one of Kay explains why very potent anti-kidney serum obtained from ducks does not produce a latent period. We have, however, heard today from Dr. Seegal that there is a difference in the localization of duck antibodies from weak sera and antibodies obtained from very potent sera. I believe this is an interesting contribution, and possibly of considerable importance in reference to this problem.

DR. WILLIAM EHRICH (University of Pennsylvania): I should like to project a slide which shows the difference in the reaction of the kidney to potent and weak sera in rats treated with anti-kidney serum produced in rabbits. The two rats were part of a lot of some 150 rats which received seven different doses (0.25-0.8 ml. of serum per 100 g. body weight) of one and the same antibody preparation.

In Figure 17 the right photo shows a lesion due to a potent serum, the left a lesion due to a weak serum. The animal represented at the right had 4+ proteinuria; it had the full-blown nephrotic syndrome. The animal represented at the left had little proteinuria. The peak of proteinuria in the latter group usually came a little later than that of the former (seventh instead of third day). Their lesion was proliferative rather than regressive. There was an increase in the number of nuclei.

These observations are in full agreement with Dr. Seegal's findings; they agree also with the experience of Drs. Sarre and Moench of Freiburg in Germany. The latter authors used young and adult rabbits and sera produced in ducks. With small doses or weak sera they got a "nephritic" lesion, while, with large doses or more potent sera, they obtained a nephrotic syndrome.

Another point which I should like to discuss is Dr. Seegal's demonstration of gamma globulin in the glomeruli.

Dr. Dixon, not long ago, showed that fibrinoid contains gamma globulin. He drew the conclusion at that time that this would indicate that fibrinoid is due to an antigen-antibody reaction. However, deposition of fibrinoid is an inflammatory process. Gamma globulin accumulates on a field of inflammation because of increased permeability of blood vessels. This is an entirely non-specific accumulation. It does not mean that antigen or antibody is involved.

The glomerulus is a very special filter. Under normal circumstances, albumin passes through, while under pathological conditions larger protein molecules may be caught in the membrane.

I wonder, Dr. Seegal, whether the antibody against aorta, which I understood you to say did not produce glomerulitis, caused fluorescence of the glomerulus merely because it was caught in the membrane, while in the other experiments an antigen-antibody union was involved as well. It is clear today that the deposition of a fluorescent gamma globulin does not necessarily indicate an antigen-antibody reaction.

CHAIRMAN HEYMANN: Is there any further discussion, or would Dr. Lange and Dr. Seegal like to have their last words?

DR. SEEGAL: I should like to make a comment concerning the latent period in rat nephritis. When we have an exceptionally potent duck anti-rat-kidney serum, the proteinuria may occur within the first day following the injection of as little as 0.3 ml. of antiserum.

Another point I should like to make concerns the relation of the latent period to the development in the rat of antibodies to duck protein. The latent period can vary from two or three days to as long as three weeks or more. Antibody to the injected duck serum reaches its maximum in about five days and rapidly declines. It was not possible in our experience to correlate the rise in serum antibody titer with the latent period of the nephritis.

DR. MITCHELL RUBIN (Children's Hospital, Buffalo, New York): Dr. Lange, when you injected the antibody from normal duck serum, what was the volume of blood containing antibodies? Was it a complete transfusion?

DR. LANGE: The rabbits got about 150 cc. of blood.

DR. RUBIN: Do you think it is comparable to the antibody the animal had produced actively?

DR. LANGE: We replaced about half of the plasma. That means that if in the serum which we gave there was a titer of 120, right after the transfusion, we had a titer of 60. If this means anything, it would mean a twofold dilution.

If I may say one thing about Dr. Heymann's comment, the purpose was not to get into an argument with Dr. Kay, or even to advance a different idea. The purpose was to show that the latent period of the duck nephritis can be completely overcome if antibodies are supplied passively right from the beginning. This means that the duck sera produce diseases to antibodies developed in the rabbit; for, if we give these in the form of gamma globulin, the rabbit will get immediate disease if its kidney is sensitized.

CHAIRMAN HEYMANN: It is good that the hypothesis advanced by Kay some years ago has been taken up again. It obviously needs some supplementation.

Could I now ask Dr. Germuth to report on his work?

C. Experimental Anaphylactic Glomerulonephritis and Human Nephritis

DR. GERMUTH: For several years we have been studying "serum sickness" type hypersensitivity and using this form of hypersensitivity to investigate mechanisms in anaphylactic reactions. We have been interested not only in the significance of the tissue lesions but also the dynamics of antigen-antibody interaction. In most experimental procedures where glomerulonephritis is produced following the injection of foreign protein, there also occurs a high incidence of necrotizing arteritis. Accordingly, this presentation will be concerned with both experimental arteritis and glomerulonephritis.

Any discussion of experimental protracted anaphylaxis must necessarily include the following topics: a) immunologic and histologic sequences during hypersensitivity, b) mechanisms by which antigen-antibody reactions produce allergic lesions and c) the relationship of the experimental lesions to human disease.

I shall begin by discussing the behavior of homologous and antigenic heterologous proteins following intravenous injection. When one injects into the blood stream of the rabbit a homologous protein such as rabbit gamma globulin, the blood clearance of this protein is found to consist of a biphasic curve [18]. The first portion of the curve, characterized by a rapid disappearance of the protein, is due to equilibration of the protein between the intravascular and extravascular fluids. This is followed in one to two days by a slower disappearance of protein at a rate such that half of the protein is removed from the blood every five days. This phase of protein clearance is presumably due to catabolism. In the case of a homologous protein, this phase of clearance continues at a semilogarithmic rate without any deviation right up to the time when the protein can no longer be detected by present methods.

In contrast to the biphasic nature of the blood clearance of a homologous protein, the injection of a heterologous protein such as crystallized bovine albumin, which stimulates the production of antibodies, gives rise to a triphasic blood clearance. The first two components are similar to those of the homologous protein clearance, while

[18] Dixon, F. J., Bukantz, S. C., Dammin, G. J. and Talmage, D. W., The Nature and Significance of the Antibody Response (A. M. Pappenheimer, Jr., Editor), Columbia University Press, New York, 1953.



Fig. 18. Necrotizing arteritis, mesentery of rabbit sacrificed 12 days after receiving 0.5 gm. bovine albumin. H. & E. x 150

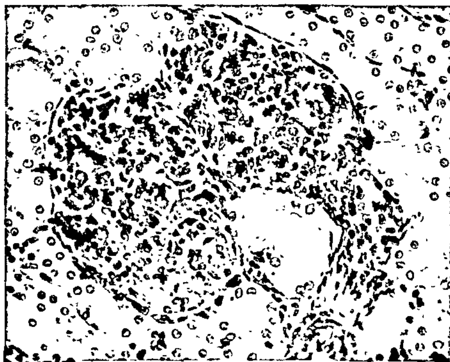


Fig. 19. Proliferative glomerulonephritis Two glomeruli showing marked proliferative changes Rabbit received 0.5 gm. bovine albumin. H. & E. x 350

the third phase is represented by rapid removal of the antigen from the blood as result of antigen-antibody union[18, 19]. This phase ordinarily begins on the sixth to the eighth day after the injection of the foreign protein. Its onset and steepness depend largely on the rate of antibody formation - a process which shows considerable variation from animal to animal. After the antigen has been completely removed, free circulating antibody appears. Free antibody is not detectable during the early formation of antibody since it must be fully saturated with the excess antigen in the body. On the other hand, free antigen cannot be detected by ordinary methods when free antibody is present.

Following the injection of a large quantity of crystallized bovine albumin into rabbits - 0.5 gram appears to be optimal - a variety of lesions are encountered which may be attributed to allergy. These include glomerular lesions, granulomatous lesions in the spleen and lymph nodes, necrotizing arteritis and endocarditis. There is good evidence that these four types of lesions are based upon the same immunologic and tissue mechanisms. In Figure 18 is shown a necrotizing arteritic lesion in the mesentery of an animal which was killed twelve days after the injection of 0.5 gm. of bovine albumin. There are present the features characteristic of acute human periarteritis nodosa (or allergic angiitis if one prefers that term). The nature of the eosinophilic fibrin-like material commonly called fibrinoid is still in doubt. It has been shown to contain fibrin[20]; but whether fibrin is the only or even the most important constituent of fibrinoid remains unknown. The glomerular lesion usually consists of a proliferative alteration characterized by a marked increase in the number of glomerular cells and swelling of the cells sometimes to such a degree that the glomerular capillaries appear bloodless (Fig. 19).

What is the relationship of the development of these lesions to the antigen clearance curve? Studies by Hawn and Janeway[21] and from our laboratory[19, 22] have shown an intimate relationship between the development of these lesions and the "immune" (third) phase of antigen elimination. In an extensive study on a large series of animals receiving 0.5 gm. of bovine albumin it was found that the lesions first appeared following the onset of antigen elimination by antibody, reached their peak incidence when the antigen had just been completely eliminated by antibody, and resolved when free antibody appeared in the circulation and no further antigen-antibody union occurred. These temporal relationships strongly suggest that these lesions are the result of antigen-antibody interaction.

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[19] Germuth, F. G., Jr., Comparative histologic and immunologic study in rabbits of induced hypersensitivity of serum sickness type. J. Exper. Med., 98: 257, 1953.

[20] Gitlin, D., Craig, J. M., and Janeway, C. A., Studies on the nature of fibrinoid in the collagen diseases. Am. J. Path., 33: 55, 1957.

[21] Hawn, C. van Z. and Janeway, C. A., Histological and serological sequences in experimental hypersensitivity. J. Exper. Med., 85: 571, 1947.

[22] Germuth, F. G., Pace, M. G. and Tippitt, J. C., Comparative histologic and immunologic studies in rabbits of induced hypersensitivity of the serum sickness type. II. The effect of sensitization to homologous and cross-reactive antigens on the rate of antigen elimination and the development of allergic lesions. J. Exper. Med., 101: 135, 1955.

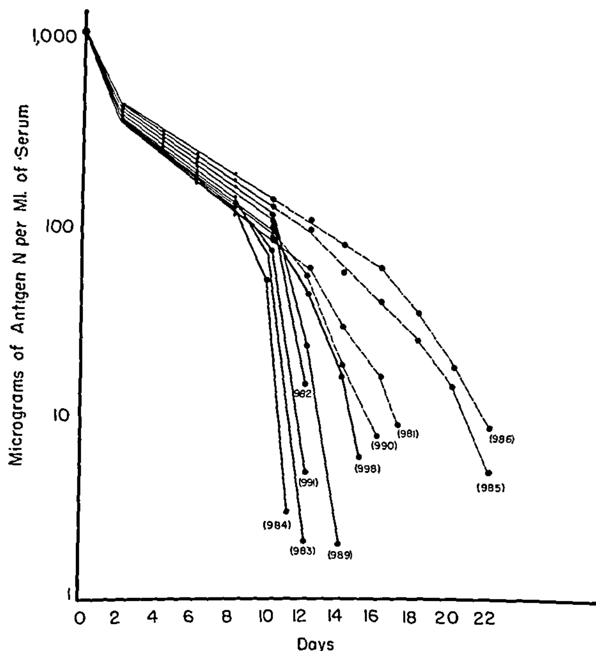


Fig. 20. (Reproduced from Germuth, F. G., Jr., Flanagan, C., and Montenegro, M. R., Bull. Johns Hopkins Hosp., 101 149, 1957.) Blood clearance and lesions with 0.5 gm. bovine albumin. Each line represents the serum antigen clearance of a single animal. Antigen clearance between injection and day 2 is due to equilibration, after day 2 until time of more rapid elimination, the clearance is the result of catabolism. Note the similarity of the equilibration and catabolic phases of antigen elimination of the individ-

It is well known that there is marked individual variation in the occurrence of allergic lesions following the administration of foreign protein[23]. In order to gain information as to whether variations in the rapidity of the immune response may be in part responsible for the presence or absence of lesions, a large scale study was carried out on the immunologic and histologic sequences following graded doses of bovine gamma globulin and of bovine albumin[24]. In Figure 20 are plotted the antigen elimination curves of twelve animals each of which received 0.5 gm. of bovine albumin. Those represented by solid lines had lesions at autopsy while those represented by broken lines did not have lesions. You can see that there is an intimate relationship between the rapidity and magnitude of the antibody responses as measured by rates of antigen elimination and the occurrence of allergic lesions. It is seen that if the antigen is still present after the fifteenth day, indicative of a poor immune response, the animals did not develop lesions. This can be explained by the fact that the production of allergic lesions requires a certain rate of antigen-antibody interaction. A similar situation, but a different temporal correlation, was seen with other doses of bovine albumin and bovine gamma globulin[24].

Mention has already been made of one type of glomerular lesion which is often encountered in experimental hypersensitivity. In addition to the ordinary proliferative glomerulonephritis, some animals, particularly those with a very rapid immune response, exhibit focal necrosis of the glomeruli similar to the human lesion of "focal embolic" glomerulonephritis (Fig. 21). When these lesions are few in number, they are found predominantly in the glomeruli adjacent to the corticomedullary junction. It is of interest that Rich[25] has recently shown that the glomerular proliferation which is seen in advanced cases of lipoid nephrosis is often confined to the glomeruli nearest the medulla. This is not to mean that lipoid nephrosis and experimental glomerulonephritis necessarily have a common mechanism. These observations suggest, however, that the glomeruli low in the cortex might be more prone to certain types of injury. A consideration of the relationship of this focal necrotizing lesion to human glomerular disease will be deferred for a moment.

The studies which have been discussed up to this point have been concerned with immunologic and histologic correlations in acute experiments; that is, in animals receiving a single injection of antigen. Now what occurs when animals are given repeated injections of bovine albumin and gamma globulin for a period of five to six weeks? In this study[26], injections of these proteins were given whenever the previous injection

[23] Rich, A. R., The pathology and pathogenesis of experimental anaphylactic glomerulonephritis in relationship to human acute glomerulonephritis. Bull. Johns Hopkins Hosp. 98: 120, 1956.

[24] Germuth, F. G., Jr., Flanagan, C., and Montenegro, M. R., The relationships between the chemical nature of the antigen, antigen dosage, rate of antibody synthesis and the occurrence of arteritis and glomerulonephritis in experimental hypersensitivity. Bull. Johns Hopkins Hosp. 101: 149, 1957.

[25] Rich, A. R., A hitherto undescribed vulnerability of the juxtamedullary glomeruli in lipoid nephrosis. Bull. Johns Hopkins Hosp. 100: 173, 1957.

[26] Heptinstall, R. H. and Germuth, F. G., Jr., Experimental studies on the immunologic and histologic effects of prolonged exposure to Antigen. I. Distribution of allergic lesions following multiple injections of bovine albumin, gamma globulin, and albumin and globulin together with special reference to the occurrence of granulomatous arteritis. Bull. Johns Hopkins Hosp. 100. 71, 1957.

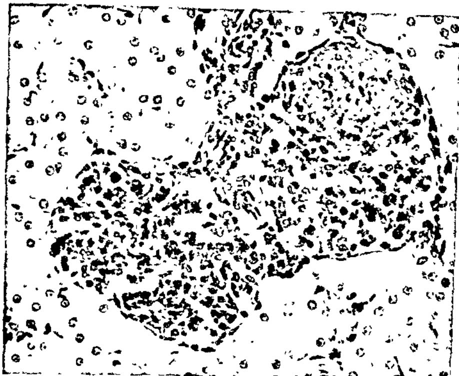


Fig. 21. Two glomeruli showing marked proliferative changes. There is focal necrosis of the glomerulus on the left. H. & E. x 350. (Reproduced from Germuth, F. G., Jr., Flanagan, C., and Montenegro, M. R., Bull. Johns Hopkins Hosp., 101: 149, 1957.

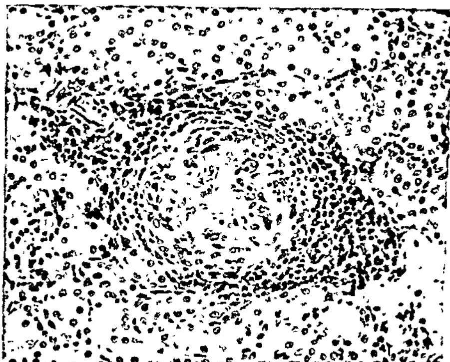


Fig. 22. Granulomatous renal interlobular arteritis. An uninvolved segment of artery can see seen in the right lower corner. Rabbit sacrificed on 35th day after receiving 6 injections of bovine albumin and 4 of globulin. H. & E. x 300 (Reproduced from Heptinstall, R. H. and Germuth, F. G., Jr., Bull. Johns Hopkins Hosp., 100. 71, 1957)

of the particular antigen had disappeared from the blood.

This schedule of antigen administration was chosen because if one treats rabbits with large injections of antigen without regard to whether there is already present a high level of antigen in the blood, "immune blackout" may be encountered - a state in which the animal no longer synthesizes antibody. On the other hand, if the antigen is given long after the previous injection of antigen when the animal has developed high levels of circulating antibody, the animal may die from anaphylactic shock. In order to follow the antigen in the blood, a capillary tube technique was devised which was capable of detecting as little as 5 to 10 micrograms of antigen in the serum. Daily determinations were done on each rabbit and whenever the test just became negative, the animal received an additional injection of antigen.

The animals which received multiple injections of antigen in this manner exhibited arterial lesions in all phases of development; acute, healing and healed. These lesions were not only present in the heart, lungs, and mesentery but were abundant in the kidney, although arterial lesions are seldom seen in the kidney following a single injection of antigen. Indeed, this has been one of the criticisms against relating the experimental lesions to human periarteritis nodosa, where the kidney is the organ most commonly involved. The arterial lesions in the kidneys of these experimental animals often were of the granulomatous or "tuberculoid" type (Fig. 22). This type of arterial lesion has been seen particularly in asthmatics dying with periarteritis nodosa or "allergic granulomatosis".

What effect did the prolonged administration of antigen have on the development of glomerular lesions? It was interesting to find that although the animals killed after a single injection of antigen showed a high incidence of glomerular lesions, those killed after multiple injections did not show progressive damage to the glomeruli as was the case with the arterial lesions. As a matter of fact, the glomerular lesion tended to resolve and the glomeruli appeared quite normal except for an occasional adhesion or scar.

The differences in the type and distribution of the allergic lesions following a brief period of sensitization on the one hand and prolonged sensitization on the other can be summarized as shown in Table 6.

Before going to the studies on the mechanism by which experimental allergic lesions are produced, it might be appropriate to discuss here the significance of the experimentally produced lesions in relationship to those of certain human disorders. The arterial, glomerular and endocardial lesions of experimental hypersensitivity have been compared to human periarteritis nodosa, glomerulonephritis and rheumatic fever. However, it is noteworthy that all of the lesions produced experimentally in the rabbit can be observed in human periarteritis nodosa alone. Further, there is every reason to believe that, just as in experimental hypersensitivity, the occurrence of a given type of lesion in human periarteritis nodosa may be influenced by the length of sensitization. Ball and Davson[27] have reported sixteen cases of periarteritis nodosa

[27] Ball, J. and Davson, J., Splenic lesions in periarteritis nodosa. J. Path. & Bact., 61: 569, 1949.

TABLE 6

EFFECT OF LENGTH OF SENSITIZATION ON THE CHARACTER AND DISTRIBUTION OF ALLERGIC LESIONS IN RABBITS

	Brief Period of Sensitization (Single injection of antigen or multiple injections up to 3 weeks.)	Prolonged Period of Sensitization (Multiple injections of antigen for periods of 3 to 7 weeks.)
Arterial Lesions:		
Stage of Development	Acute (followed by healing and healed)	Acute, healing and healed
Character of Active Lesions	Inflammatory and necrotizing	Inflammatory, necrotizing and granulomatous
Distribution	Heart, lungs, mesentery	More widespread with extensive involvement of kidneys
Glomerular Lesions:	High incidence of acute proliferative and necrotizing lesions	No prominent lesions; occasional glomerular crescents, adhesions and scars
Splenic Lesions	High incidence of granulomatous lesions in Malpighian bodies	No prominent lesions

with respect to the occurrence of glomerular lesions. A review of their data shows that of seven cases of periarteritis nodosa exhibiting only acute arterial lesions, all showed the presence of necrotizing glomerulonephritis. However, in the nine cases showing healing and healed arterial lesions, indicative of a more prolonged disease state, glomerular lesions were present in only two instances.

It is therefore clear, from experimental work and study of human autopsy material, that the necrotizing and proliferative glomerular lesions seen in rabbits are part of a generalized disease process involving blood vessels varying in size from capillaries to the larger arteries. The glomerular lesions produced by experimental hypersensitivity under conditions which often promote the development of arterial lesions must be closely related if not identical pathogenetically to the glomerular alterations of human periarteritis nodosa. As stated previously, the glomerular lesions of experimental hypersensitivity have often been compared to the glomerulonephritis in humans following streptococcal infection. Although post-streptococcal glomerulonephritis may be found in allergy, the possibility exists that glomerulonephritis as an anatomical entity might result from a variety of injuries. The role of hypersensitivity in post-streptococcal glomerulonephritis therefore awaits clarification.

One of the most important problems in the field of hypersensitivity is the understanding of the mechanism by which the lesions of hypersensitivity are produced. There are, of course, many reasons for believing that the lesions which develop following the injection of foreign proteins are indeed due to antigen-antibody interaction. The most important of these is the temporal correlation between the development of lesions and the "immune phase" of antigen elimination. However, up until recently, there was still

Relationship between the precipitin curve (Ba-anti Ba system) and the capacity of supernatant fluid to produce shock.

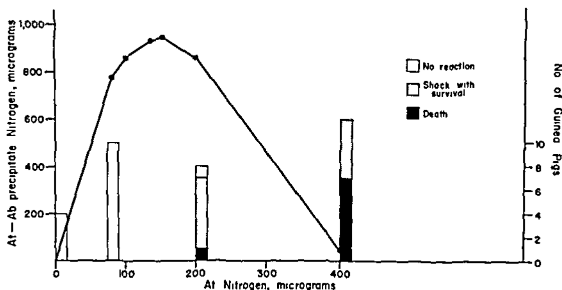


Fig. 23. (Reproduced from Germuth, F. G., Jr. and McKinnon, G. E., Bull. Johns Hopkins Hosp., 101: 13, 1957)

Ba = bovine serum albumin, Anti-Ba = rabbit antiserum to Ba

lacking the demonstration that the lesions could be produced by the passive transfer of antibody in the presence of antigen. In unpublished experiments carried out with Dr. Abou Pollack, the passive transfer of serum sickness has now been accomplished. Rabbits were injected with 0.5 gm. of bovine albumin and, 24 hours later, infused intravenously with potent antiserum. The rate of infusion of antiserum was so chosen that the antigen in the blood would be completely eliminated before the development of lesions due to an active immune response to the injected antigen. Control animals receiving antigen were infused either with saline or normal rabbit serum. In the animals infused with antiserum the rate of antigen elimination was proportional to the rate of antibody infused. The molecular ratio of antibody to antigen for complete elimination of the antigen was calculated to be approximately 1 to 1. Thus less antibody was required for clearance of the antigen in vivo than for maximal precipitation of antigen in the test tube where the molecular ratio is approximately 2 to 1. Lesions were observed only in the antibody-infused animals and were quite like those seen in active sensitization, consisting of proliferative glomerulonephritis and necrotizing arteritis. The results of these experiments will be published in detail in the near future.

For some time, we have been concerned with the mechanism by which antigen-antibody reactions lead to lesions of hypersensitivity. It had been postulated that the lesions were produced by the combination of free antibody with antigen in the tissues. However, since the allergic lesions are initiated while excess antigen is present in the circulation it seemed unlikely that free antibody as such could get to the tissue sites without prior combination with antigen. In one of our early studies the possibility was presented that antibody might be transported to the tissues in the presence of excess circulating antigen in the form of soluble antigen-antibody complexes[19]. It is well known that soluble antigen-antibody complexes are produced in vitro when antibody is added to excess antigen. Recently Sternberger and his colleagues[28] and Weigle[29] have clearly demonstrated the presence of soluble antigen-antibody complexes in the sera of animals receiving foreign protein. Our purpose was to determine whether soluble antigen-antibody complexes could elicit anaphylactic reactions in the normal animal. Since anaphylactic shock in the guinea pig is a most sensitive indicator of anaphylactic reactions, the effect that soluble antigen-antibody complex might have on the guinea pig was first tried. To tubes containing 1 ml. of antiserum were added varying amounts of antigen. After precipitation, the supernatant fluid from each set of tubes prepared with identical amounts of antigen was pooled and injected in 2 ml. volumes into normal guinea pigs. The results of a typical experiment, in this case the bovine albumin-antibovine albumin system, are presented in Figure 23. None of the animals receiving whole serum or the supernatant fluid from antigen-antibody mixtures containing excess antibody exhibited ill effects. However, the majority of guinea pigs receiving comparable volumes of supernatant fluid from antigen-antibody mixtures prepared with excess antigen and therefore containing soluble antigen-antibody complexes showed signs characteristic of anaphylactic shock. At autopsy, the animals which died rapidly showed the marked pulmonary emphysema typical of anaphylactic shock[30].

[28] Sternberger, L. A., Maltaner, F. and DeWeerdt, J., Estimation of circulating antibody-antigen complex. J. Exper. Med., 98: 451, 1953.

[29] Weigle, W. O., In vivo antigen-antibody complexes, Fed. Proc. 16: 437, 1957.

[30] Germuth, F. G., Jr., and McKinnon, G. E., Studies on the biological properties of antigen-antibody complexes. I. Anaphylactic shock induced by soluble antigen-antibody complexes in unsensitized normal guinea pigs, Bull. Johns Hopkins Hosp. 101: 13, 1957.

It was important to determine whether the shock produced by the supernatant fluid obtained from antigen-antibody mixtures was indeed due to soluble antigen-antibody complexes or whether it was due to the liberation of histamine or proteolytic enzymes by antigen-antibody interaction in vitro as supposedly observed throughout the entire precipitin curve. In order to further determine the immunologic basis for the anaphylactogenic activity of the supernates, the effect of precipitation of the excess antigen and antigen-antibody complexes by the addition of specific antiserum to supernates on the anaphylactogenic activity of the supernates was investigated. When this procedure was done, it was found that the supernates no longer produced shock. Time will not permit further discussion of the numerous experiments carried out to investigate the biologic activity of soluble antigen-antibody complexes. The evidence indicating that soluble antigen-antibody complexes are responsible for shock elicited by the supernatant fluid of antigen-antibody mixtures is as follows: 1) But for minor exceptions, only supernates from antigen-antibody mixtures prepared in antigen excess are capable of eliciting shock. 2) Shock can be produced by mixtures of purified antibody (gamma globulin) and excess antigen. 3) The further addition of excess antibody to supernates originally prepared in antigen excess removes the anaphylactogenic properties. 4) Soluble antigen-antibody complexes, prepared by the dissociation of washed immune precipitates by alkali in the presence of additional antigen, produce shock. 5) The amount of antibody contained in soluble antigen-antibody complexes capable of producing shock is similar to the amount required in passive anaphylaxis produced by the simultaneous injection of antigen and antibody.

Not only can antigen-antibody complexes induce anaphylactic shock in normal guinea pigs but these materials are also capable of eliciting a skin reaction of the Arthus type when injected intracutaneously. Of course, our main objective was to determine whether the lesions of serum sickness could be obtained by the intravenous infusion of soluble complexes. In preliminary experiments carried out with Dr. Abou Pollack and Dr. George McKinnon it was found that such infusion would produce proliferative glomerulonephritis and granulomatous lesions in the spleen which were morphologically similar to those of active serum sickness. Unfortunately, up to the present time, necrotizing arteritis has not been encountered. The failure to produce this lesion might be due to difficulties in technique. This is being further explored.

It is clear that anaphylactic reactions do not require the actual interaction of antigen and antibody in the tissues but may be produced by preformed antigen-antibody complexes acting on susceptible cells. Whether the effect of antigen-antibody complexes is triggered by the addition of complement is presently under study.

Finally, I should like to mention briefly studies on anaphylactic shock in the rabbit. Our attention was directed to this study by the experiments done on the production of lesions by repeated injections of antigen. Following a single injection of foreign protein, all of the lesions ordinarily attributed to serum sickness were obtained with exception of the so-called anaphylactic pneumonitis which was first described by Rich[31] and related by this investigator to rheumatic pneumonia. However, this lesion was repeatedly

[31] Gregory, J. E. and Rich, A. R., The experimental production of anaphylactic pulmonary lesions with basic characteristics of rheumatic pneumonitis. Bull. Johns Hopkins Hosp. 78. 1, 1946.

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[28] *Sternberger*, L. A., Maltaner, F. and DeWeerdt, J., Estimation of circulating antibody-antigen complex. J. Exper. Med., 98: 451, 1953.

[29] Weigle, W. O., In vivo antigen-antibody complexes, Fed. Proc. 16: 437, 1957.

[30] Germuth, F. G., Jr., and McKinnon, G. E., *Studies on the biological properties of* antigen-antibody complexes. I. Anaphylactic shock induced by soluble antigen-antibody complexes in unsensitized normal guinea pigs, Bull. Johns Hopkins Hosp. 101: 13, 1957.

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[31] Gregory, J. E. and Rich, A. R., The experimental production of anaphylactic pulmonary lesions with basic characteristics of rheumatic pneumonitis. Bull. Johns Hopkins Hosp. 78: 1, 1946.

seen in rabbits which received multiple injections of antigen, particularly when successive doses of antigen produced symptoms of anaphylactic shock. In view of the large amounts of antigen and antibody necessary to produce shock in the rabbit, it occurred to us that the amorphous pulmonary thrombi described by Rich might be due to *in vivo* antigen-antibody combination. Accordingly fluorescein-tagged antigens were injected into highly sensitized animals. All of the animals which developed symptoms or succumbed to anaphylactic shock showed widespread obstruction of the pulmonary capillaries by fluorescent immune precipitates [32]. This obstruction was of such a degree as to readily account for the right-sided heart failure which is so characteristic of shock in the rabbit. This finding is important not only in the understanding of anaphylactic shock in the rabbit but also demonstrates the extreme caution one must employ in relating experimental lesions to human disease.

It is clear that much remains to be learned concerning the role of hypersensitivity in disease. Certainly one of the most important problems is that relating to the pathogenesis of the tissue damage produced by antigen-antibody interaction.

CHAIRMAN HEYMANN: Thank you, Dr. Germuth. This paper is open for discussion.

DR. EHRICH: I should like to ask Dr. Germuth whether he thinks serum sickness is an example of the anaphylactic type of allergy, or of the wheal and erythema type, or both.

DR. GERMUTH: I cannot answer that.

DR. CHARLES A. JANEWAY (Harvard Medical School, Department of Pediatrics): I think these experiments of Dr. Germuth's are terribly interesting. The way he has kept working on the problem is very commendable. The conception that the soluble complexes may produce lesions, and possibly the symptoms of certain aspects of hypersensitivity reactions, is a very interesting one.

We had an unfortunate opportunity to study serum sickness in the human under somewhat unusual circumstances during the war. We were studying blood substitutes, and gave people very large doses of plasma protein substitutes which were chemically very homogeneous but unfortunately proved to be powerful antigens. We unfortunately saw quite a lot of serum sickness. Several things were apparent.

First, it was possible to have serum sickness from very minute traces of globulin impurities in the bovine albumin. These could be demonstrated by the appearance of positive skin tests, or as pure gamma globulin, or by the disappearance curve of the antigen.

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[32] McKinnon, G. E., Andrews, E. C., Jr., Heptinstall, R. H., and Germuth, F. G., Jr., An immunohistologic study on the occurrence of intravascular antigen-antibody precipitation and its role in anaphylaxis in the rabbit, Bull. Johns Hopkins Hosp. 101: 258, 1957.

The uniqueness of our experiment was the fact that we were dealing basically with almost a single, pure antigen in the immunologic sense. Whereas people dealing with serum sickness in the days when they used large amounts of foreign protein were dealing with mixtures of many different protein species.

The results which one would have predicted from our animal experiments, which were similar to those of Dr. Germuth but were not in such great detail, were just as he said, antigen and antibody were never seen in the circulation at the same time. Presumably, if the antigen was responsible for the lesions and the disease, as well as for antibody reacting with it, once the antibody had reacted with all the available antigen, the patients should get well and the excess antibodies should appear in circulation.

This occurred with several of the patients; but we had the interesting experience of observing several other patients who were every bit as sick, and reacted just as violently, but in whom the process stopped short of completion; and the antigen did not all disappear. Its curve, which had been descending steeply, straightened out, then proceeded as it would had no serum sickness occurred, but at a level which would be the equivalent of three months later in somebody who had not reacted. These individuals never developed positive skin tests, and never developed positive precipitin tests in the serum for this antigen; yet, certainly, they were clinically as ill by any criterion we could apply.

I do not know whether Dr. Germuth's observations help to explain our observation, I suspect they do. In view of Dr. Germuth's work, it is possible that the serum sickness actually occurs in the time of antigen excess. You still have antigen present in the circulation; there is no free antibody although, clearly, it is being formed. As for these people, I suppose, we still cannot explain what happened, except that perhaps, for some strange reason, they had lost their capacity at that time to respond further to this injury, and therefore remained in the zone of antigen excess; antibodies were no longer being formed to produce any complexes and, therefore, there were no longer any symptoms.

DR. STONE. Many years ago, on the basis of very few experiments, a folklore was laid down that anaphylaxis was due to some mysterious tissue-sensitizing antibody and, in fact, that circulating antibody was inhibitory of anaphylaxis.

People who worked in the field of hypersensitivity really always had this curious hypothesis crop up, and did not quite know what to do about it.

We can look now at other species of animals in which the work on anaphylaxis has gotten less attention because of the wonderful reaction of the sensitized guinea pig to its homologous antigen, for example, work done on anaphylaxis in mice. Anaphylaxis is due to precipitating antibody; and animals die of acute anaphylactic shock while having high titers of homologous circulating antibodies in their serum. This is probably true of other species of animals, such as the rabbit and rat, which also have not been used too frequently for anaphylactic experiments.

CHAIRMAN HEYMANN: Any further comments or questions?

Dr. Germuth, will you answer?

DR. GERMUTH: *As you know, for a long time anaphylactic shock in the rabbit was attributed to spasm of the pulmonary vessels. If you saw in the lungs of a patient the number of thrombi that we see in the shocked rabbit, you would have little doubt that death was due to acute embolization. I would think that in vivo antigen-antibody precipitation plays a very great role in anaphylactic shock in the rabbit. This would explain the right-sided heart failure which is so common.*

CHAIRMAN HEYMANN: I have been told that we do not belong to a union, and that we do not have to stop at 5 p.m. sharp. If it meets with your approval, we might continue until six o'clock, I hope not longer.

Dr. Rothbard will discuss the renal lesions due to anticollagen serum.

D. Renal Lesions Due to Anticollagen Serum

DR. SIDNEY ROTHBARD (The New York Hospital): During the past several years, my colleague, Dr. Robert F. Watson, and I, at the New York Hospital-Cornell Medical Center, have been interested in the general biologic properties of collagen, one of the characteristic constituents of connective tissue.

Previous reports[33, 34] from this laboratory have shown by both in vivo and in vitro methods that collagen is antigenic. Purified preparations of collagen from the tail tendons of rats, little altered from the native state, are soluble in dilute acetic acid. Repeated intraperitoneal injections of collagen solutions over a period of time induce complement-fixing antibodies in rabbits. Experiments were designed to show by immunologic methods, enzymatic analysis, and electron microscopy that these antibodies were specifically directed to the collagen fibers. Complement fixation and precipitin tests have shown that the antibody is not directed to contaminants, such as rat serum; and by inhibition tests it has been shown that tissue polysaccarides do not enter into this reaction. Collagenase, an enzyme which attacks only gelatin and collagen, disintegrated the collagen and destroyed its serologic activity.

By electron microscopy it was possible to obtain more direct information concerning the specificity of the antibody. Purified solutions of collagen upon neutralization develop fibers which have the characteristic banding and periodicity of native collagen. Upon the addition of rabbit serum containing complement-fixing antibodies to these solutions, the fibers were not formed, but rather macromolecular units or a globular precipitate. Apparently, the antibody coats these macromolecules and thus prevents fiber formation. If one adds normal rabbit serum instead of antibody, the usual fiber is formed.

[33] Watson, R. F., Rothbard, Sidney, and Vanamee, Parker, The Antigenicity of Rat Collagen, J. Exp. Med., 99: 535, 1954.

[34] Rothbard, Sidney, and Watson, R. F., Antigenicity of Rat Collagen. Reverse Anaphylaxis Induced in Rats by Anti-Rat Collagen Serum. J. Exp. Med., 103: 57, 1956.

One can easily see the characteristic banding and periodicity of the native fiber obtained from the rat tail tendon. Collagen in solution in acetic acid, under the electron microscope at a magnification of about x35,000, appears amorphous. Upon neutralization one finds the fiber reconstituted and showing the characteristic periodicity of the native collagen. The electron micrographs provided strong evidence that the antibody was directed toward the collagen fiber itself and supported the concept that collagen is antigenic.

Rabbit serum containing antibodies against rat collagen, when injected intravenously into the rat, causes an immediate reverse anaphylactic shock, often resulting in death. Normal rabbit serum or serum containing antibodies to collagen from tunica or carp swim bladder was without effect.

The shock had all the clinical and laboratory characteristics of anaphylaxis, such as thrombocytopenia, leukopenia with a rebound leukocytosis, lowering of the complement level, delayed blood coagulability, and the development of a refractory state.

The pathological findings were characteristic of anaphylaxis in this animal species as reported by Parker and Parker [35] in direct anaphylaxis and by Smadel and Swift [36] in reverse anaphylaxis. The most striking change was in the small intestine. Patchy and diffuse hemorrhages extended from the pylorus to the cecum, and the lymphoid tissue of Peyer's patches was covered with petechial hemorrhages. Blood was often found in the lumen of the small intestine. Histological examination showed more widespread vascular changes than seen in the gross. Erythrocyte thrombi were found in numerous small veins and capillaries. Often the erythrocyte outlines were lost, resulting in a homogeneous hyaline acidophilic appearance. In the intestine, the smaller mesenteric vessels were occluded, resulting in infarction of large areas. The alveolar walls of the lungs were thickened, congested, and contained many of these erythrocyte thrombi. Large numbers of polymorphonuclear leukocytes were sequestrated in the capillaries of the widened alveolar walls.

The reacting substance in these sera containing the antibodies was found in the gamma globulin fraction of the serum. Furthermore, by appropriate absorption with preparations of collagen, the reacting substance was removed and no shock occurred, an indication that the rabbit anti-rat collagen serum is specifically directed to a substance, apparently collagen, in the rat.

Since systemic anaphylaxis is one of the most sensitive indicators of the combination of antigen with antibody, these studies, made in vivo, confirmed and extended the in vitro studies of the antigenicity of collagen; but, more important, it gave us a clue by which we might be able to study in the animal host tissue injury by specific antibodies.

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[35] Parker, J. T., and Parker, F., Jr., Anaphylaxis in the White Rat. J. Med. Research, 44: 263, 1924.

[36] Smadel, J. E., and Swift, H. F., Reverse Anaphylaxis in Rats with Special Attention to Kidney Damage. J. Immun., 32: 75, 1937.

Careful examination of various sections from the animals injected with either lethal or multiple sublethal doses revealed no apparent specific injury to collagen by the methods employed. Since one of the aims of these studies was to attempt to demonstrate injury to collagen by specific antibody, a variety of experiments was done. During the course of these studies, a group of rats was immunized with normal rabbit serum incorporated in the Freund adjuvant. When antibodies to the normal rabbit serum were at a high level, rabbit anti-rat collagen serum was injected intravenously and the animals sacrificed after an appropriate interval. An unusual form of diffuse glomerular lesions was found in the kidneys.

The kidneys of these rats were enlarged and pale with smooth surfaces and tense capsules. On hemisection, no gross abnormalities were found, but on microscopic study a generalized diffuse injury to the glomeruli was remarkable. The glomeruli were enlarged and relatively avascular with marked proliferation of epithelial cells and, to a lesser extent, endothelial cells, reducing the capillary lumina; on occasions, mitotic figures were seen. The capsular and capillary basement membranes were thickened, shredded, and fused to each other. In large numbers of the altered glomeruli, giant cells of the Langhans type, resulting from the fusion of the proliferating epithelial cells, were prominent features. A few of the glomeruli contained focal masses of hyaline-like material, and in others, large amounts of pink-staining protein were found in Bowman's space. Polymorphonuclear leukocytes and fibrin thrombi were absent from the glomerular tufts. Many of the proximal and distal convoluted tubules, as well as the collecting tubules, contained precipitated protein and hyaline casts. There was little change in the interstitial tissue or in the walls of the medium or large sized vessels.

In contrast, I will describe a representative control glomerulus before the injection of the provocative anti-collagen serum. The representative glomerulus was obtained from a rat sacrificed one week after the last injection of normal rabbit serum incorporated in the Freund adjuvant. The rat at this time was immunized and antibody to the rabbit serum was at its peak level. Rabbit anti-rat collagen serum had not been given. The noteworthy features were the patent capillaries, normal outlines of the basement membranes, a single layer of epithelial cells in Bowman's capsule, and the lack of any fusion of the capillary tuft. However, there was an increase in cellularity, particularly of the endothelial cells with dark nuclei.

For comparison, various types of glomerular lesions found in rats which have been immunized with normal rabbit serum incorporated in the Freund adjuvant and subsequently injected intravenously with rabbit anti-rat collagen serum, will be described.

The most common type of injury to the glomeruli seen in the experimental rats, where the glomerular tuft is swollen and ischemic, includes diffuse proliferation of the epithelial cells, with mitotic figures, and, to a lesser extent, that of the endothelial cells. The proliferation often is so extensive that Bowman's space is obliterated. Adhesions bind the parietal and visceral basement membranes, which are swollen and shredded. Fibrin thrombi, necrosis, and polymorphonuclear leukocytes are not seen. The exudative component of inflammation which often accompanies the proliferative phase is very meager. A noteworthy feature is the minimal amount of blood in the glomerular capillaries.

Another common lesion in the glomerular tufts involves prominent, bizarre giant cells, apparently formed from the coalescence of the glomerular epithelial cells, as well as the proliferation of the epithelial cells. In addition, the capillary channels are obliterated with almost complete loss of normal structure of the tuft. The proximal convoluted tubules contain precipitated protein and casts in their lumina, but the epithelial cells of the tubules are intact.

One also commonly finds a distorted glomerular tuft with intense proliferation of epithelial cells, containing a giant cell, fused and shredded basement membranes, ischemia, and also hyaline-like masses.

A less common abnormality, occurring in addition to the enlarged glomerular tuft, giant cells, and avascularity, results in tubules with dilated lumina filled with massive hyaline casts. Interstitial tissue changes were not seen in this instance.

We have observed an unusual instance in which apparently one of the Langhans giant cells, contained in the glomerulus, has slipped from the glomerulus into the lumen of a proximal convoluted tubule.

An uncommon type of lesion may be observed in which Bowman's space is dilated and filled with pink-staining protein-rich fluid. This same fluid is seen in the lumina of the nearby convoluted tubules. Interstitial involvement is absent. The ischemia, proliferation of cells, and thickening of the basement membranes are present.

Control rats immunized in the same way with normal rabbit serum incorporated in the Freund adjuvant and then injected intravenously with normal rabbit serum, high-titered rabbit anti-group A streptococcus, anti-hemocyanin, or anti-fish collagen sera failed to develop any of these renal glomerular lesions.

Microscopic examination of the experimental and control animals revealed granulomata with epithelioid cells and multinucleated giant cells, without necrosis, in the lungs and regional lymph nodes draining the sites of the injected adjuvant. However, the glomerular renal lesions were found only in the rats injected with the antibody to rat collagen.

For example, a microscopic section of a regional lymph node draining a site injected with the adjuvant revealed a lack of caseation, large numbers of plasma and epithelioid cells, lymphocytes, and the multinucleated giant cells. Lipid material being phagocytized by the giant cells was noted.

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[37] Freund, Jules, The Mode of Action of Immunologic Adjuvants, Adv. Tuberc. Res., 7: 130, S. Karger, Basel New York, 1956.

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new sites of antibody formation. The Freund adjuvant contains lipids and mycobacteria, each of which may induce the granulomata. Satisfactory antibody titers could not be attained in the rats unless the adjuvant was employed, but use of incomplete adjuvant, i.e., without tubercle bacilli, did not affect the subsequent development of nephritis. Thus, tubercle bacilli can be excluded as playing a role in this disease.

To determine whether the normal rabbit serum fraction carried in the rabbit anti-rat collagen serum was essential for the induction of this form of glomerulitis, rats were immunized with normal chicken or horse serum incorporated in the Freund adjuvant and then were injected with the rabbit anti-rat collagen serum. Nephritis was found, indistinguishable from that observed in rats immunized with normal rabbit serum. The possibility that this lesion is just serum nephritis resulting from the interaction between rabbit globulin and its corresponding antibody appears to be unlikely, because rats immunized with normal rabbit serum in the adjuvant develop circulating antibodies and do not show any apparent renal disease on subsequent injection with normal rabbit serum. This experiment was also done with chicken and horse serum, and no glomerular lesions were observed.

Examination of the urine in several rats revealed proteinuria, microscopic hematuria, and granular and hyaline casts. Nitrogen retention was shown by study of the blood.

The question arose whether this form of nephritis was in any way related to the nephrotoxic nephritis in rats described by Masugi[38], Smadel[39], and Seegal[40]. Studies have been done with Dr. Beatrice Seegal of the Department of Microbiology at Columbia University to attempt to establish this point. Microscopic sections of these two forms of nephritis from comparable rats were studied, and it was concluded that they differed. The nephrotoxic nephritis lesion is characterized by the exudative component of inflammation with fibrin thrombi in the glomerular capillaries, infiltration with polymorphonuclear leukocytes, and necrosis; whereas, the renal lesion in the present study is more extensive and shows more proliferative cellular change in the glomeruli. Although bizarre giant cells were noted in some of the glomeruli of the nephrotoxic nephritis, those found in the present study were more extensive, distinct, and consistently observed. In vitro immunologic studies revealed the antigens to differ. Dr. Seegal provided us with antisera against rat whole kidney and against rat glomeruli, as well as whole rat kidney antigen. Our own preparation of soluble rat tail collagen and its corresponding antibody were available. With these materials it was possible to do cross complement fixation tests to determine whether the collagen antigen is related to the nephrotoxic antigen.

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[38] Masugi, M., Uber das Wesen der spezifischen Veranderungen der Niere und der Leber durch das Nephrotoxin bzw. das Hepatotoxin. Beitr. path. Anat., 91: 82, 1933.

[39] Smadel, J. E., Experimental Nephritis in Rats Induced by Injection of Anti-Kidney Serum. I. Preparation and Immunological Studies of Nephrotoxin. J. Exp. Med., 64: 921, 1936.

[40] Seegal, B. C., and Bevans, Margaret, The Production of Glomerulonephritis by Immunologic Methods. J. Chron. Dis., 5: 153, 1957.

The results of studies of the complement fixation reactions employing these antigens and their corresponding antibodies, show that the collagen antigen does not fix complement with antisera against either the rat kidney or rat glomeruli, whereas ample fixation to a titer of 1:32 is noted with antibody to collagen. Also evident, but less clear-cut was the fact
the presence of antibody to
antibody. The fact that rat
probably due to collagen in
reaction occurred with purified rat tail collagen antigen and antibody to rat kidney or glomeruli because the collagen present in the rat kidney was not made soluble when injected into the rabbit for the preparation of the nephrotoxic antibody. Nevertheless, it is possible to conclude that the antigen used for the production of nephrotoxic nephritis differs from the collagen antigen employed in the present study.

Although the precise mechanism for the production of this renal lesion is not entirely clear, all these experiments show that the rabbit anti-rat collagen serum is essential. One hypothesis is worthy of mention: that two antigen-antibody systems are involved. In the first system, the normal rabbit serum incorporated in the Freund adjuvant induces antibodies to this antigen, and the subsequent reaction alters the renal glomeruli sufficiently for the second antigen-antibody reaction, i.e., anti-rat collagen serum with a collagen antigen in the renal glomeruli, to produce the nephritis.

These experiments probably raise many more questions than they answer. Nevertheless, a method is available by which tissue injury by specific antibody may be studied in the animal host.

CHAIRMAN HEYMANN: Thank you, Dr. Rothbard. The paper is open for discussion.

DR. GOODMAN: Have you injected rats with Freund's adjuvant alone, without incorporating any normal rabbit serum in it, for the original injections before putting in your anticollagen serum?

DR. LANGE: Have you used the antigen without Freund's adjuvant?

DR. ROTHBARD: Yes, Dr. Lange, in many experiments, but they were not detailed in our presentation. Multiple injections of normal rabbit serum over prolonged periods were given to rats and subsequent injections of anti-rat collagen serum were given intravenously but no lesions were demonstrable. We attempted to follow the pattern of Kay's experiment by multiple injections of rabbit anti-rat collagen serum intravenously into normal rats, but no renal abnormalities were found. This could be explained by the fact that the rat is a relatively poor antibody former. Passive transfer of antibodies to normal rabbit serum was also employed. High-titered serum was prepared in rats immunized with normal rabbit serum in Freund adjuvant. This antiserum was then injected into rats which were given the anti-rat collagen serum. Again no lesions could be found. Because the multinucleated giant cells might have been due to the mycobacteria in the Freund adjuvant, experiments were done without these bacteria, but the renal lesions were consistently observed nonetheless.

Your question, Dr. Goodman, can be answered partly from the last point. The incomplete Freund adjuvant was as effective as the whole adjuvant, but no experiments as yet have been done with adjuvant not containing some form of normal serum. It is apparent that the antibody to collagen has something to do with this lesion.

Obviously, there are numerous other experiments which have to be done; but it appeared appropriate to let you know at this time that another form of nephritis in animals can be induced by antibody, besides the nephrotoxic nephritis or serum nephritis.

DR. HENRY BARNETT (Albert Einstein College of Medicine, Yeshiva University): Would you want to say a little more about the changes in the blood in the disease which is produced?

DR. ROTHBARD: Not many studies have been done along this line, but the BUN does rise in the early phases of the disease. Rats now have been followed for seven months, and more chronic changes are being noted in the microscopic sections of the kidney lesions. There has been no evidence of a clinical nephrotic syndrome.

CHAIRMAN HEYMANN: How about edema?

DR. ROTHBARD: As I said, no nephrotic syndrome has appeared as yet.

DR. LANGE: Would you want to call these lesions nephritis?

DR. ROTHBARD: You can call it anything you like, Dr. Lange, but you will agree these are not normal glomeruli. The precise anatomical nature of this lesion interests us less than the mechanism by which it is brought about. What appears to be important is that these lesions are suggestive of injury caused by the collagen antibody.

DR. JULES FREUND: The nature of the morphology is very interesting to me, because it reminds one of the reaction to something which is not easily soluble.

One could argue that these lesions are derived from the mycobacteria; but that is wrong, because you have some controls in which the same adjuvant was injected, and there are no such lesions. It is your combination. You do get these interesting lesions which remind one of sarcoid or noncaseating tubercles, or lesions around things like cotton or silica, and so on. So then I would assume that there is something there which is not easily soluble. I wonder how long these lesions would stay. I would expect that they would persist for a very long time, but do you have any information on that?

DR. ROTHBARD: Seven months. I mentioned that we have sacrificed animals at that time, and the lesions had progressed to a point of fibrosis. Strangely enough, the giant cells in the glomeruli had disappeared, but you would expect that with the fibrotic change in the glomeruli.

DR. FREUND: But this type of lesion distinguishes it very much from Masugi nephritis, or any other.

DR. ROTHBARD: Oh, yes. I think Dr. Seegal, who is sitting alongside you, would agree wholeheartedly that, when we compared the two types of lesions, we were impressed by the differences. The proliferative type of lesion in the present study as compared to the exudative form in the nephrotoxic (Masugi) nephritis. You cannot always separate, as you know, Dr. Freund, the exudative from the proliferative phase. Even in tuberculosis, they go hand in hand. One will go a little higher than the other. But in these present experiments one is impressed by the high degree of proliferative changes with, not a complete, but almost a complete lack of the exudative components.

DR. FREUND. Do you suspect some insoluble material there? Maybe Dr. Germuth would have something to add.

DR. GERMUTH: Yes, I think your point, Dr. Freund, is well taken. I would only say, however, that we have seen several cases of it with nephritis where the lesion in the glomerulus has been a granulomatous lesion. What has gone on there I do not know; however, it has been recorded.

DR. ROTHBARD: I went over Dr. Rich's photomicocraphs time and again, and yours; and I have seen Dr. Seegal's slides. She, too, has described bizarre giant cells but did admit - and I think she would again - that the multinucleated giant cells are more prominent in these current studies. I was just curious about your statement.

DR. GERMUTH: When I said that, I meant in humans, not in our experimental work.

DR. ROTHBARD. Oh, I know of one paper, too[41], and I looked up the photomicrographs. I admit they were present, but not within the glomeruli.

DR. GERMUTH. They can be within the glomerulus; I have seen several cases myself.

DR. JACK METCOFF: Would you be good enough to tell us what the source of the collagen is, and whether you have any idea as to what point on the fiber or fibril the antibody fixes?

DR. ROTHBARD. In all the studies the collagen was obtained from rat tail tendon of Whelan strain hooded rats. This is the strain that Smadel and Farr[42] employed at the Rockefeller Institute for Medical Research in New York in their studies on the nephrotoxic nephritis. Unfortunately, there are very few colonies of this strain left in the country.

Where the collagen fiber is acted on by the antibody is unknown to me.

DR. EHRICH: I should like to support Dr. Rothbard. These lesions do not occur in serum sickness.

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[41] McManus, J. F. A., and Kornsby, A. T., Granulomatous Glomerulonephritis Associated with Polyarthritis. Arch. Path., 52: 84, 1951.

[42] Smadel, J. E., and Farr, L. E., The Effect of Diet on the Pathological Changes in Rats with Nephrotoxic Nephritis. Am. J. Path., 15: 199, 1939.

However, I have seen them once, not in serum sickness, but in five animals which had been treated with large doses of staphylococcal vaccine[43]. We interpreted these reactions as a *primary response* of the organism to bacteria or their products rather than an allergic phenomenon.

DR. GERMUTH: Do you know what happens if you take collagen, as you have done, and add another antigen-antibody system to it?

DR. ROTHBARD: We have not done that; but that is a good point.

I am glad someone has not brought up the question as to whether there is collagen in the normal glomerulus.

DR. GERMUTH: I have wanted to ask you about the experiments on Masugi nephritis by Cruickshank and Hill[44] *where they take an antibody, put a fluorescent* dye on it, and have stained reticulin throughout many tissues, including tendon. Are you certain you are dealing with collagen and not with reticulin?

DR. ROTHBARD: What is the difference between reticulin and collagen? Within *the limitations of the electron microscope they are identical. A pertinent study on this* subject was published by Irving and Tomlin[45]. The silver staining of reticulin which is considered characteristic of this fiber is shown to be due to a complex of ground substance. Collagen can be given the argyrophilic properties of reticulin by providing the fibers with hyaluronic acid, blood pasma, or other blood proteins. This report strongly indicates that *mature collagen consists of reticular fibers in a ground substance* medium.

DR. GERMUTH: I only thought it was interesting, if their work is correct, that they did not stain collagen fibers as we know them.

DR. ROTHBARD: The reticulin fiber probably would stain in the same way as collagen if one removed the polysaccharide from the reticulin.

Several reports[46] have indicated that no collagen can be shown in the normal glomerulus, but it is possible that by *removal of the ground substance the typical banding* of collagen might be observed by electron microscopy and the characteristic staining *demonstrated*.

Immunologic methods, which are more specific, could be used to determine this point.

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[43] J. Exp. Med., Vol. 49, plate 30, 1929.
[44] Cruickshank, B., and Hill, A. G. S., Histochemical Identification of a Connective Tissue Antigen in the Rat. J. Path. and Bact., 66. 283, 1953.
[45] Irving, E. A., and Tomlin, S. G., Collagen, Reticulum and Their Argyrophilic Properties. Proc. Roy. Soc. London, Series B, 142: 113, 1954.
[46] Farquhar, M. G., Vernier, R. L., and Good, R. A., Studies on Familial Nephrosis. II. Glomerular Changes Observed with the *Electron Microscope*. Am. J. Path., 33: 791, 1957.

DR. GERMUTH: That is why I thought Cruickshank's and Hill's paper was very interesting.

DR. ROTHBARD. It was. D. G. Scott[47] employed the immuno-histologic technic of Coons and found two distinct antigens, reticulin and basement membrane, in the renal glomerulus, as well as the basement membrane of the tubules.

Also more interesting, I think, is a very important point made by Goodman, Greenspon, and Krakower[48] who found that there was an antigen common to both parietal capsular membrane of glomeruli and collagen fibrils from cornea and tendon.

CHAIRMAN HEYMANN: Before you go back to your seat, I would like to say that, as Dr. Barnett indicated, quite a few of us are very much interested in the biochemical alterations in this disease. If you ever have some spare serum, many of us will be very happy to run the chemistries, whatever you want, and send the results back to you.

Dr. Goodman, will you kindly present your material?

E. Search for Anti-Kidney Antibodies in Patients

DR. GOODMAN: It is late in the afternoon; and I will be short and show no slides. I am in the unenviable position of talking about negative data, knowing full well that there are at least two slides in the audience which show positive data on the subject I am going to discuss.

It is obvious from the preceding discussions that in human renal disease antibodies to kidney may not be detectable even if the renal disease has an immune basis. First of all, the disease may not even be produced by an antibody against kidney; it may be produced by an antibody against an antigen, perhaps bacterial, which has localized at the kidney. In this case one would certainly not expect to find any circulating antibody against kidney tissue. Secondly, even if the immune mechanism (assuming that there may be one) responsible for the renal disease does involve antibodies against kidney tissue, those antibodies may either be absorbed by the tissue, and therefore not be circulating, or they may be circulating, but not detectable by the still somewhat crude methods we must use. Nevertheless, there have been many reports in the literature which suggest that antibodies to kidney tissue are present in some patients with renal disease. I will not describe these in detail, but by the use of complement fixation or adsorption of kidney extracts on collodion particles, reactions between patients' sera and suspensions or extracts of human tissue have been found by some investigators, including Dr. Lange[49]. Voisin[50] has written a detailed survey of the work in this

[47] Scott, D. G., A Study of the Antigenicity of Basement Membrane and Reticulin. Brit. J. Exp. Path., 37: 178, 1957.
[48] Goodman, Morris, Greenspon, S. A., and Krakower, C. A., Antigenic Composition of the Various Anatomic Structures of the Canine Kidney. J. Immun., 75. 96, 1955.
[49] Lange, K., Gold, M., Weiner, D., and Simon, J., J. Clin. Invest. 28: 50, 1949.
[50] Voisin, Guy, Cytotoxic Auto-antibodies, a clinical and experimental critical study. 30th Congres Francais de Medecine, Alger, 1955. Paris, Masson, 225-264, 1955.

field including references to work which failed to demonstrate such auto-antibodies. In addition, the demonstration by Mellors[51] and his associates that gamma globulin coated the capillaries of the glomeruli in certain patients with renal disease lends support to an immune basis for their renal lesions. The work of Dr. Rose (who is here) and Dr. Ernest Witebsky, has shown that the sera from some patients with chronic thyroiditis contain circulating proteins which may be antibodies to thyroid extract. Many other reports[50, 52] show that in animals auto-immunization with certain tissues (central nervous system, testicular, or thyroid) is possible, and that lesions in these tissues can be produced by auto-immunization. In view of all these reports, Dr. Baxter and I decided to see what we could find in the way of circulating antibodies to kidney tissue in the sera of patients with renal disease.

In our work we have chosen to use the complement-fixation test and the tanned red-cell hemagglutination test. In the latter test the red cell is exposed to dilute tannic acid and is then capable of absorbing protein substances. When the tanned, antigen-coated red cells are suspended in serum containing antibody, the red cells will agglutinate and settle in positive patterns of agglutinated red cells. It is a sensitive method for translating an antigen-antibody reaction into a visible reaction.

We have used tanned cell hemagglutination tests, coating the tanned red cells with saline extracts of human kidney which were obtained at autopsy. We tested these kidney extract-coated tanned red cells against the sera of twenty patients with the nephrotic syndrome whom we have seen on our service. Four of these patients did not respond to steroid therapy, and three are patients who died, after failing to respond to steroid therapy. We were given two additional sera from patients presumed to have acute nephritis. I could summarize our results with these sera by saying that we have not found any evidence by complement-fixation or tannic-acid hemagglutination tests of antibodies which would react with the extracts of human-kidney tissue. We stopped at this point, because we have engaged in other experiments to show that we are actually coating the red cells with human-kidney antigens.

I want to spend a few minutes discussing the one positive reaction we have found because we have been rather interested in it. This was in the serum of a patient with lupus erythematosus. It has been reported that some patients with lupus erythematosus have sera containing high titers of complement-fixing antibodies against tissue antigens[53]. In the three or four sera we have tested, we have not found such high titers, but one serum had a titer of about one to ten, a very weak titer, when tested with red cells coated with a saline extract of human kidney. We wondered if we were detecting a reactant similar to that responsible for the LE cell phenomenon. We thought that the gamma globulin in our patient's serum might be reacting with a nucleoprotein in the saline extract (although 0.15M saline is not supposed to extract nucleoproteins), in the same way the gamma globulin reacts with what is presumably a nucleoprotein in the nucleus to change the nucleus so it can then be phagocytized to form an LE cell.

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[51] Mellors, R. C., Ortega, G. L., and Holman, H., J. Exp. Med. 106: 191, 1957.
[52] Witebsky, E., Rose, N. R., Terplan, K., Paine, J. R., and Egan, R. W., J.A.M.A. 164: 1439, 1957.
[53] Gajdusek, D. C., Nature, 179: 666, 1957.

We then extracted liver as well as kidney by Chargaff's method[54] in order to get out nucleoprotein. First the tissue was ground in a Waring blender with 0.15M saline. The saline extract was discarded and the precipitate was washed again with saline. Finally the nuclei were ground in a Waring blender with distilled water and were extracted overnight with distilled water. A clear extract was obtained by ultracentrifugation. We coated tanned red blood cells with dilutions of the distilled-water extract of nuclei of liver and (in other experiments) kidney.

With this procedure, we found good titers of agglutinins, or antibodies if you wish, against tanned cells coated with the extract of nuclei. I brought in a positive test showing the pattern of agglutination of the extract-coated red blood cells when they are suspended in the patients' serum diluted up to 1:1280.

I will just mention three aspects of this phenomenon which have interested us.

1. What sera will show the reaction? Three patients with systemic lupus erythematosus have had titers of 1:340, 1:640, and 1:1280. The patient whom I originally described was admitted with renal involvement, the others do not have an obvious renal lesion. Sera from two other patients who are in clinical remission, but do have a positive LE test, do not show this reaction. However, we have tested thirty other sera from patients with many conditions including nephrosis, rheumatic fever, hyperglobulinemia and other disease states, and have not found any positive reaction in any of these sera.

2. The second question is, what is the protein in the serum which is reacting with the coated, tanned red cell? Dr. John Fahey has been kind enough to do a starch electrophoresis analysis of the serum. It is definitely the gamma globulin, which produces the positive reaction with the coated tanned red cell.

3. Finally, what is the "antigen", if you care to call it that, what is the substance with which we are coating the red cell? Our extract is obtained by using a method for extraction of nucleoprotein. The active substance is not in saline extract of the nuclei or cytoplasm, or it is only present in very low concentrations. The activity of the extract is destroyed by desoxyribonuclease, although it is also destroyed by trypsin. The extract has a greater absorption at 260 than at 280 in the Beckman spectrophotometer using the ultraviolet. We think a nucleoprotein is present. Whether this is actually the active substance we do not know.

I will apologize for presenting our material in this preliminary fashion, but we wanted to tell you about the progress of our work to date. So far we have not succeeded in finding autoantibodies in patients with renal disease in our preliminary experiments. In the patients with lupus erythematosus I think it is reasonable to believe that we are measuring something akin to the gamma globulin which is responsible for the LE cell phenomenon. Whether our extract contains all the nuclear substances with which the serum will react or only one of such substances we do not know. And whether the gamma globulin which reacts with the extract of liver nuclei has anything to do with the pathogenesis of the disease, of course, is a question which cannot be answered at present.

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[54] Chargaff, E., and Davidson, J. N., in The Nucleic Acids, Academic Press, New York, p. 313, 1955.

CHAIRMAN HEYMANN: May I supplement your statements, Dr. Goodman, with results that we obtained last year in collaboration with Dr. Rothenberg[55]?

The question may be raised whether one uses the right antigen. At the time we had a model which we believed could test the suitability of the antigen to increase the hemagglutination titer. An anti-human kidney serum obtained from rabbits of a certain titer served that purpose. When the antigen was altered by trypsin digestion or by other procedures, we were able to increase the titer, let us say from 1:80,000 to 1:10 million; coating tannic acid treated red blood cells with such prepared antigen solutions, did not alter our results obtained with sera of children suffering from the nephrotic syndrome. They still remained negative.

DR. GOODMAN: Even though those same cells would react with anti-human-kidney rabbit serum.

CHAIRMAN HEYMANN: Yes, up to 1:800,000 or 1:10 million.

DR. GOODMAN: We have had the same experience.

CHAIRMAN HEYMANN: I believe that Dr. Pfeiffer was first to tell me that he would like to present some additional data. Dr. Pfeiffer!

DR. E. E. PFEIFFER (Frankfurt, Germany): Dr. Bruch and I[56] have been concerned with the demonstration of antibodies against human kidney tissue since 1950, when Franz Volhard still was the head of our department, and currently since Ferdinand Hoff has taken over our department. We got positive reactions mainly in cases of progressive chronic glomerulonephritis, using the collodion-particle technique. Dr. Hoff in the early forties with a certain serological technique[57] and Dr. Lange in 1949 with the collodion-particles, also got positives. The positive reactions have been more consistent in hypertensive than in nephrotic patients. However, we were not happy either with the method or the reproduction of the serological findings in the individual case, therefore, we switched over to the complement-fixing test. However, the results were even more disappointing. With four different methods of complement fixation, we had absolutely negative results[58].

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[55] Rothenberg, M. B., Stavitsky, A. B., and Heymann, W., In-vitro Evaluation of Nephrotoxic Sera in Experimental Nephrosis, Pediatrics, 18: 455, 1956.

[56] Pfeiffer, E. F. u. H. E. Bruch, Autoantikoeper gegen menschliches Nierengewebe, Verh. Dtsch. Ges. Inn. Med., 56: 189, 1950.
Ueber den Nachweis von Autoantikoerpern gegen menschliches Nierengewebe bei Nieren - und Hochdruckkranken mit der Kollodium-Partikel-Reaktion, Dtsch. Arch. Klin. Med. 144: 613, 1952.

[57] Hoff, F. and Wendlberger: Quoted in F. Hoff, Medizinische Klinik, Stuttgart, Georg Thieme, p. 147, 1948.

[58] Spielmann, W., Pfeiffer, E. F., and Bruch, H. E., Zur Problematik des Nachweises von Autoantikoerpern gegen gesundes und krankes Nierengewebe bei Nierenkranken mittels Komplementbindungsreaktionen, Z. Ges. Exp. Med. 123: 236, 1954.

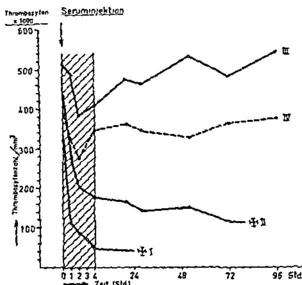


Fig. 24. Thrombocytopenic reaction in a rabbit after injection of 10 cc. (curve I) and 5 cc. (curve II) of serum of a patient with idiopathic thrombocytopenic purpura with positive serological findings in vitro, of 10 cc. of absorbed patient serum (curve III) and of 10 cc of normal serum (curve IV).

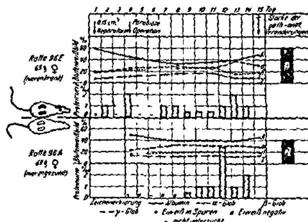


Fig. 25. Transfer of experimental glomerulonephritis by means of parabiosis Electrophoretic pattern of blood proteins and proteinuria in parabiotic rats, from which the upper one suffered on experimental nephritis, induced by injection of nephrotoxic rabbit serum 3 days before the parabiotic junction Development of proteinuria and changes of the blood proteins in the originally healthy animal (below) on the 6th day after the unification

On some, we also tried the tannic-acid hemagglutination test; and we were no more satisfied with it.

Therefore, we attempted to find another way to approach this problem because, of course, even with constantly reproducible titers of anti-kidney antibodies the pathogenic significance of these antibodies remains undetermined.

In some hematological diseases, however, commonly considered of auto-allergic origin, there is a method of proving the pathogenic effect of an auto-antibody against blood cells, visible under the microscope in vitro, by transfusing the antibody-bearing blood or serum of the patient to healthy human volunteers or certain suitable animals.

Figure 24 demonstrates such a biological test with the serum of a patient with an idiopathic thrombocytopenic purpura that contained in vitro antibodies against human as well as rabbit platelets[59].

After injecting 10 cc. of the patient's serum into a rabbit the thrombocytes in the rabbit dropped from $321{,}000/mm^3$ to $112{,}000/mm^3$ within one hour, to $48{,}000/mm^3$ after 4 hours; and the rabbit died after 24 hours due to thrombosis of the caval vein (Curve I). This method was suggested by Miescher[60] in Switzerland and the first transfusion experiments in human beings were done in this country by Harrington[61]. The injection of 5 cc. of the patient's serum was followed by a minor decrease of the circulating thrombocytes in the rabbit and death occurred only after 84 hours (Curve II). The injection of the patient's serum, initially absorbed with human or rabbit platelets, as well as of the serum of normal subjects, resulted in only a slight drop of thrombocytes lasting only a few hours (Curves III and IV).

Unfortunately, in diseases of parenchymatous tissues, like glomerulonephritis in advanced stages, such a simple biological method is not available. As a rule, a single transfusion of a nephritic individual's blood does not induce significant signs of nephritis in the healthy recipient.

Therefore, visualizing the different nature of a fixed parenchymatous tissue antigen, compared with the mobile circulating blood cell antigen, in 1954 we decided to perform the transfusion experiment in another way, that is, by establishing a continuous blood transfusion from the nephritic individual to a healthy recipient, by means of parabiosis[62].

[59] Pfeiffer, E. F., Spielmann, W., u. Ditschuneit, H., Die klinische Bedeutung des Nachweises von Antikoerpern gegen Thrombocyten bei thrombocytopenischer Purpura, Dtsch. Med. Wschr. 81: 735, 1956.
The clinical significance of the Anti-platelet Factor in Thrombocytopenic Purpura, German Medical Monthly, 1: 229, 1956.

[60] Miescher, P., Cruchaud, S., and Hemmeler, G., Helv. Med. Acta, 19: 434, 1952.

[61] Harrington, W. I., Minnich, V., Hellingsworth, I. V., and Moore, C., J. Lab. Clin. Med., 38: 1, 1951.

[62] Pfeiffer, E. F., Schoeffling, K., Sandritter, W., Schroeder, J., Steiger, H., Nephritis der Ratte: Die Uebertragung durch kurzdauernde Parabiose, Z. Ges. Exp. Med. 124: 471, 1954.



Fig. 26 Transfer of experimental glomerulonephritis by means of parabiosis: Histological changes in the kidneys of the two parabionts, the clinical findings of which were given in slide 25, demonstrated in a single glomerulus from the primarily nephritic animal (left) and the originally healthy rat (right).

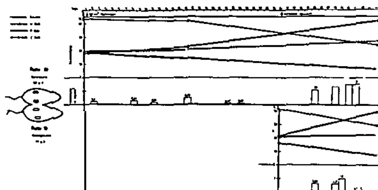


Fig. 27. Transfer of experimental glomerulonephritis by means of parabiosis Blood proteins and proteinuria in a nephritic parabiotic pair, in which the experimental nephritis in the primarily nephritic parabiont (upper rat) lasted 30 days before the parabiosis with a healthy rat (lower animal) was performed.

The experimental procedure is given in Figure 25: Mild nephritis was induced in the first animal by the intravenous injection of a small amount of rabbit anti-rat-kidney antiserum.

If the induced nephritis was too severe because of large amounts of the nephrotoxic rabbit-antiserum used, the rats did not tolerate the parabiotic operation.

After the injection we waited 3-5 days until proteinuria and changes in the blood proteins demonstrated that the nephritis had been established; then, the parabiotic junction of a nephritic and a healthy rat was performed. Three to five days later, when the blood shunt between the two animals started to work, and 8-10 days after the injection of the nephrotoxic rabbit serum into the first animal, proteinuria and changes in the electrophoretic pattern of the blood proteins were observed in the originally healthy parabiont too. At the end of the experiment there were - and this is easily understandable by the blood shunt between the two animals - there were often the same quantitative changes in the blood proteins in both parabionts. Sometimes, the proteinuria was even greater in the originally healthy animal than in the primarily nephritic one.

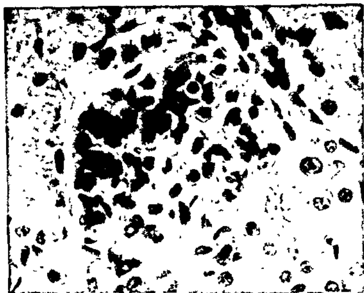
We have sacrificed all these animals on the 12th to 15th day after the parabiotic junction, in order to prevent the so-called "parabiosis-intoxication" or "-allergy", which has been described after a longer duration of the parabiosis.

Figure 26 demonstrates the histological changes in the pair whose clinical findings we have just considered. In both animals there was an increased number and a swelling of the endothelial cells of the glomeruli, swelling of the basilar membrane and adherence of the glomerular loops. In the tubules, hyaline thrombi were present and also hyaline droplets in the epithelial cells.

These changes have been classified as 3+, if the increase in the number of the endothelial cells was marked, as 2+ if less, and as 1+, if only swelling of the endothelial cells and little droplet formation was observed. Table 7 gives the summary of the pathological findings, according to this classification.

Out of 238 white rats of a single strain, but not inbred, which have been used in this experiment, 68 have been finally evaluated, 13 nephritic and 15 control parabiotic pairs and 6 other controls in which only the clinical findings have been checked to date. As you can see, 2+ reactions occurred only 3 times in the controls, while 3+ changes, i.e., marked signs of an inflammatory process, were never seen in this group, also no significant proteinuria nor striking changes in the blood proteins. In the nephritic parabiotic pairs, however, 3+ changes were noted twice and 2+ changes occurred 7 times in the originally healthy parabionts.

There are three objections to the conclusion that these results show that autoantibodies, supposedly developed in the primarily nephritic parabiont, damage kidney tissue in the originally healthy one: First, as Dr. Heymann kindly suggested to me by letter two years ago, the possibility was not excluded that the heterologous rabbit antibodies injected into the first nephritic animal were shunted later to the parabiotic partner and the original heterologous antibodies in reality, produced the nephritis in the originally healthy animal.



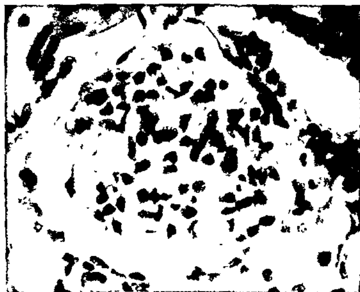


Fig. 28 Transfer of experimental glomerulonephritis by means of parabiosis: Histological changes in the kidneys of the parabiotic couple, the clinical findings of which have been given in Fig. 27. The lesions have been more progressive in the kidneys of the primarily nephritic rat (left glomerulus) than in the originally healthy one (right glomerulus.

TABLE 7

SUMMARY OF THE MORPHOLCGICAL CHANGES IN THE KIDNEYS

OF THE NEPHROTIC PARABIOTIC AND THE CONTROL RATS

Intensity of Reaction	Nephritic parabiosis		Controls	
	prim. nephr. animal	prim. healthy animal	left animal	right animal
0	-	1	6	4
1+	2	3	8	9
2+	5	7	1	2
3+	6	2	-	-
No. of rats:	13	13	15	15

Therefore, Dr. Schoeffling, Dr. Sandritter, Dr. Treser, Dr. Kraus and I[63] repeated these experiments but waited 30 days between the induction of the primary nephritis and the parabiotic union.

As you can see from Figure 27, however, the results were the same as in the former experiments, but only the histological changes differed between the two parabiotic partners (Fig. 28).

As you can see in the glomerulus taken from a kidney of the primarily nephritic animal, the changes have been more progressive in this rat; the glomerular loops are already attached to the wall of Bowman's capsule, while in the kidneys of the originally healthy partner the lesions have not progressed to the same degree, presumably because here the nephritis lasted only a short time compared with the duration of the disease in the first animal.

The second objection was raised by the successful transplantation of a kidney from a healthy identical twin into the nephritic twin, as performed at the Peter Bent Brigham Hospital in Boston, by Merrill and associates[64]. From these studies it could be concluded that possibly no specific autoantibodies but the genetic differences between the two parabiotic partners were responsible for the nephritis in the originally healthy animal. Therefore, we repeated our experiments with a strain of rats inbred over 59 generations[63]. However, the results were exactly the same as before (Fig. 29).

[63] Pfeiffer, E. F., Die Bedeutung des Parabioseversuches fuer das Autoallergieproblem, Verh. Dtsch. Ges. Allergieforschg. and Verh. Dtsch. Ges. Ophthalmologie (Joint Meeting), 8-10 Sept. 1957, Heidelberg (in press).
Pfeiffer, E. F., Schoeffling, K., Sandritter, W., Treser, G., and Kraus, E.: to be published.

[64] Merrill, J. P., Murray, J.E., Harrison, J.H., and Guild, W. R., Successful Homotransplantation of the Human Kidneys between Identical Twins, J. Am. Med. Assn. 160: 277, 1956.

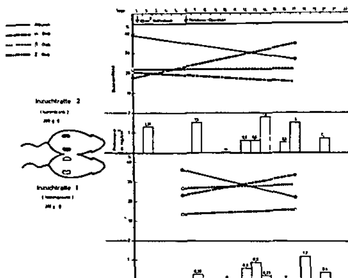


Fig. 29 Transfer of experimental glomerulonephritis by means of parabiosis. Electrophoretic pattern of blood proteins and proteinuria in an inbred parabiotic pair which has been united on the fifth day after the injection of the nephrotoxic serum in the upper, primarily nephritic partner. Development of proteinuria and changes of the blood proteins in the originally healthy animal (below) occurred as in the not-inbred parabiotic rats (see Figs. 24 and 27).



Fig. 30 Morphological findings in the eye of a rat which has been given rabbit anti-rat-eye-antiserum. Hyperemia of the iris and the choroid tissue, infiltrations of round cells in the uvea, detachment of the retina. The lens has become opaque.

According to Dr. Merrill, with whom I recently discussed our experiments, this could have been expected, since in human twins, the nephritic kidneys are removed after the transplantation while they remain in situ in our parabiotic rats. Experiments are now in progress in which the kidneys of the primarily nephritic rat are removed at the time of the parabiotic junction.

The third objection was raised by the possibility that no anti-kidney-auto-antibody but some pathological protein was developed as a consequence of the nephritic process. This protein was filtered by the kidneys of the healthy partner and produced the transfer of the nephritis from one animal to the other. Therefore, we tried to repeat our parabiotic experiments but with inflammatory processes induced in other organs than the kidneys.

The first organ process supposed to be transferred by means of parabiosis was allergic encephalomyelitis and we learned that Dr. Freund and Dr. Lipton already had done this successfully in rats[65]. However, we were not able to confirm their findings because we failed to induce allergic encephalomyelitis in the first animal by using Freund's adjuvant technique. I am very happy to see Dr. Freund here today and I want to take advantage of my stay here, Dr. Freund, to ask you if you can supply me with some of your rats of that strain which is susceptible to develop allergic encephalomyelitis. In the meantime, however, we decided to use another organ, the eye. With Dr. Thiel, Dr. Menk, Dr. Otto and Dr. Kurus from the Ophthalmological Department of our University and Dr. Schoeffling, Dr. Treser and Dr. Bachrach from our own hospital[66] we studied the effect of an experimentally induced allergic panophthalmitis in the healthy eyes of parabiotic partners. The experimental procedure was analogous to the renal experiments, including the induction of the panophthalmitis in the first rat by the injection of rabbit anti-rat-eye-serum which was produced by the sensitization of the rabbits with whole-rat-eye-tissue in order to get lesions of the whole eye.

Within 24 hours after the injection of this anti-eye-serum, hyperemia of the vessels of the iris and the retina as well as hemorrhages were observed in 24 of 29 rats. I should like to mention that these changes could be easily noted clinically by ophthalmascopic examination. In some of these animals we found detachments of the retina too.

The next slide (Fig. 30), a section of the whole eye, demonstrates the morphological findings in one of these animals after the injection of the rabbit antiserum. The retina is detached. There is hyperemia of the iris and the choroid tissue, and infiltrations of round cells also have appeared in the uvea. The lens has become opaque.

And now the histological findings in the eyes of the primary healthy parabionts which were united with the ophthalmitic animals 5-7 days after the injection of the anti-rat-eye-serum. The lesions appeared in these experiments as in the nephritis studies

[65] Lipton, M. M., and Freund, J., The Transfer of Experimental Allergic Encephalomyelitis in the Rat by Means of Parabiosis, J. Immunol., 71: 380, 1953.

[66] Menk, G., Experimentelle Untersuchungen ueber organspezifische Veraenderungen am Auge, Verh. Dtsch. Ges. Allergieforschg. and Verh. Dtsch. Ges. Ophthalmologie (Joint Meeting), 8-10 Sept. 1957, Heidelberg (in press). Pfeiffer, E. F., Thiel, R., Menk, G., Schoeffling, K., Otto, X., Kurus, X., Treser, G., and Bachrach, I.: to be published.



Fig. 31 Transfer of experimental panophthalmitis by means of parabiosis Cornea of an originally healthy rat which has been united in parabiosis with a primarily ophthalmitic partner. Vascularization of the cornea as sign of a previous inflammation.



Fig. 32. Transfer of experimental panophthalmitis by means of parabiosis Hyperemia of the iris of an originally healthy rat which has been united in parabiosis with a primarily ophthalmitic partner.

5-8 days after the parabiotic junction. They were partially visible with the ophthalmoscope, they were demonstrable clearly in the tissue sections after sacrifice.

Figure 31 shows vascularization of the cornea as a sign of a previous inflammation and Figure 32 demonstrates hyperemia of the iris; and infiltration in the iris and in the ciliary body is shown in Figure 33. Figure 34 demonstrates marked infiltration of round cells around the retinal vessels, which is better shown in Figure 35 at higher magnification.

There were no pathological findings in the eyes of 10 parabiotic control couples, united without any treatment. Furthermore, pathological changes were completely absent in brain, heart, liver, spleen and kidneys of the originally healthy parabionts. Thank you.

CHAIRMAN HEYMANN: Dr. Liu, I believe you also had some data to present.

DR. CHARLOTTE LIU: Thank you for the opportunity for letting me talk.

We collected sera from 148 hospitalized patients. Their sera were tested by Boyden's hemagglutination test[67]. The test was done by treating the sheep blood cells with 1:10,000 tannic acid. The antigens for coating on the tanned sheep cells were the high speed supernate of a 2 per cent saline kidney extract[68] and the tryptic digest of kidney by Cole's method[69]. These were human kidneys obtained immediately or within 3-4 hours after death. The nitrogen content in these preparations was 1-2.5 mg.% for the kidney digest and 0.1-0.15 mg.% for the kidney supernate.

In each test, we had at least one negative serum from a normal individual and a positive serum from a rabbit immunized against a 20 per cent human kidney homogenate. Control tubes were set up with tanned sheep blood cells without coating with any antigens in the lowest dilution of each serum and the tanned sheep cells coated with the antigen in the diluent which had no testing sera. These control tubes all read negative.

The results of the hemagglutination test from the 148 sera are summarized in Table 8. These titers were to the kidney digest. The kidney supernate gave titers the same or one or two dilutions lower than those with the kidney digest. The majority of sera from acute nephritic and nephrotic patients had titers at or above 1:40. The chronic nephritic patients were essentially negative. Some of the sera from patients with acute infections had titers above 1:40. The rabbit anti-human-kidney serum had a hemagglutinin titer of 1:800 against kidney supernate and 1:6,400 against kidney digest.

I was interested to hear that Dr. Goodman found a positive test in lupus erythematosus. I have not included it in this table, but I have done one and its hemagglutinin titer was 1:160.

[67] Boyden, S. V., The Adsorption of Proteins on erythrocytes Treated with Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera, J. Exper. Med. 93: 107, 1951.
[68] Adler, F. L., Strain-Specificity in Tissue Antigens of Mice, J. Immun., 74: 63, 1955.
[69] Cole, L. R., Cromartie, W. J., and Watson, D. W., A Specific Soluble Substance Involved in Nephrotoxic Nephritis, Proc. Soc. Exper. Biol. and Med., 77: 498, 1951.

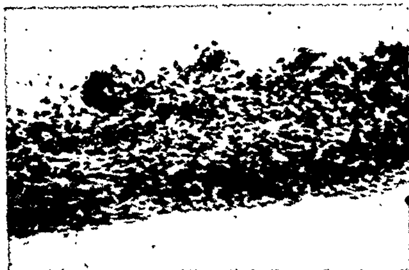


Fig. 33: Transfer of experimental panophthalmitis by means of parabiosis: Infiltration of the iris (as well as of the ciliary body) of an originally healthy rat which has been united in parabiosis with a primarily ophthalmitic partner.

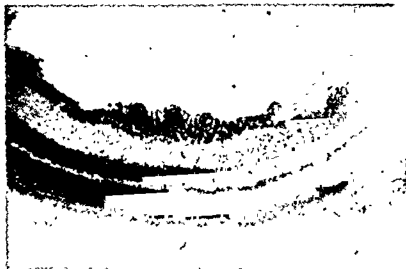


Fig. 34. Transfer of experimental panophthalmitis by means of parabiosis Marked perivascular infiltration in the retina of an originally healthy rat which has been united in parabiosis with a primarily ophthalmitic partner.

We have also followed the titers in 24 nephrotic patients during their cortisone treatment. Their hemagglutinin titers did not show any definite drop until two or three weeks after cortisone treatment. In these 24 patients, there were 91 per cent having titers at or above 1:40 before the cortisone was started. At the third or fourth week of treatment, only 37 per cent still had titers of 1:40 or above.

In conclusion, it seems that there were some anti-kidney antibodies demonstrable by this hemagglutination test. However, these titers in patients are low. Dr. Goodman pointed out that these antibodies would be bound with the antigen in the kidney, as soon as they were produced. This may explain the finding of low titers of antibodies in their sera..

TABLE 8

DISTRIBUTION OF ANTI-KIDNEY HEMAGGLUTINATION TITERS OF HUMAN SERA BY BOYDEN'S TECHNIQUE

Disease	Number of subjects	<1:10	1:10	1:20	1.40	1:80	1:160	1:320	1:640 or more
Acute nephritis	35	5	5	3	9	6	6	0	1
Nephrotic syndrome	54	2	4	1	14	7	14	5	7
Chronic glom. nephritis	12	9	2	1					
Term. nephrotic synd.	7	5	2						
Normal	16	15	1						
Infectious disease	14	4		1	4	2	1	2	
Rheumatic fever	10	3	4		2		1		
Total	148								

Since we also found anti-kidney antibodies in the sera of patients with infectious disease, the significance of these antibodies in respect to renal disease is uncertain. We have run some of the positive human sera and the rabbit anti-human-kidney serum against human organs other than kidney; such as lung, liver and heart. They did show cross-reactions to these other organs. However, these titers were generally lower than those for kidney. These organ cross-reactions are similar to the findings of the organ antigen content in nephrotoxic serum nephritis in rats[70]. It is probably a reflection of the complexity of antigens and the distribution of these specific antigens in various organs.

CHAIRMAN HEYMANN: Thank you, Dr. Liu.

[70] Nephrotoxic Serum Nephritis in Rats. I. Distribution and Specificity of the Antigen Responsible for the Production of Nephrotoxic Antibodies, J. Exper. Med., 104: 467, 1956.



Fig. 35 Transfer of experimental panophthalmitis by means of parabiosis Same slice as demonstrated in Fig. 34 in higher magnification. Perivascular infiltration in the retina

DR. LANGE: I am in a very peculiar position. In 1946 we proposed that anti-kidney antibodies were present; and we believed at that time that they were the causative mechanism of nephritis and nephrosis. We explained the lack of their appearance in certain cases by the fact that they may be all absorbed onto the kidney.

Since then we have changed our mind and think that these antibodies, which were so beautifully demonstrated here today, are actually secondary to the destruction of kidney tissue by the primary deposit. There is at first a coating of the glomerulus by a specific substance against which an antibody is formed which then destroys kidney tissue as in the animal experiments. The destroyed, degraded kidney tissue then leads to production of an antibody against this degraded kidney. We now think it is this latter antibody we described in 1946. The experiments reported here today nicely clarify this concept.

DR. MILTON RAPOPORT: (Children's Hospital, Philadelphia): There were some preliminary studies which I think Dr. Liu said nothing about. These were attempts to produce antibodies to homologous organs on which she spent a great deal of time. She used kidney enzymatically digested, or saline extracts of kidney, and immunized rabbits with their own kidneys mixed with cocci. You get antibodies; but these antibodies were demonstrable not only with kidney tissue, but also with an oil emulsion and with a great many other tissues. I think our trite conclusion was that we had partially immunized the rabbit to himself, there was something in the kidney which every other organ shared. Perhaps some of the studies reported here today are of this nature.

DR. N. R. ROSE (Buffalo, New York): The hypothesis that various diseases might be caused by auto-immunization is a time-honored one; and I think, by now, there is hardly a disease left which has not been accused of being caused by an auto-antibody.

But I think it is necessary to establish this; and there are certain criteria which we should fulfill in order to implicate definitely an autoantibody in the production of disease. I think the most important one is to have some idea as to what the antigen involved is, and how it might possibly function as an autoantigen.

The experimental diseases, such allergic encephalomyelitis, testicle immunization to produce a spermatogenesis, and more recently certain thyroid diseases, we feel, represent rather special antigens which apparently are somewhat isolated from the body in an immunological *sense*. Specifically, they are not available autogenously, as a rule, so that the usual immunological tolerance which the body would naturally develop toward its normal constituents does not occur. Therefore, these are foreign substances; and, if they are made available to the antibody-forming sites by some accident, it is conceivable that they would stimulate autoantibody formation.

In the case of thyroid, of course, one has to have a rather unique situation where the thyroid globulin is stored in sacs within the thyroid tissue, and in somewhat isolated form.

The question arises, do other organs of a similar nature have such antigens which are hidden from the blood stream?

We know of none; and here we come to the problem, in doing these tests, to which Dr. Goodman referred; that is, what antigen can we use in trying to demonstrate such an antibody in the patient's serum?

It seems fairly unlikely that we would be able to demonstrate such antibodies with an antigen which is normally accessible to the blood stream. Therefore, one is tempted either to find antigens which are isolated anatomically or which are isolated by some chance of solubility. That is the great interest in Dr. Goodman's demonstration of a reaction with a nuclear protein. Perhaps also this sort of reasoning might be applied to collagen and other substances which are not normally soluble and, in that sense at least, somewhat isolated.

One also must be quite cautious, I think, in labeling a serum factor an antibody. In order to do this, we are really confronted with the necessity of establishing two things: One is that the antibody arises by immunization, and that it is specific in its function. The easiest way of doing this would be to immunize an experimental animal artificially with the antigen which we believe is involved, to show that the antibody results from immunization, and that it is perfectly specific in its function.

Again, we feel that this can be done and has been done in certain instances, thyroid, for example. For that reason, we are content to call this factor present in the serum of certain patients with thyroid disease an autoantibody.

DR. CONRAD M. RILEY (Presbyterian Hospital, New York): I was particularly interested in Dr. Pfeiffer's report of his parabiotic rats and the transfer of something from one parabiont to the other, which produced proteinuria at least.

We had a similar idea; and Dr. Ruth Alice Davis, who was working with me a few years ago, performed quite a few experiments in which she collected blood from acutely nephrotic rats (induced by nephrotoxic serum), pooled it, and gave normal rats a replacement transfusion. I cannot remember the volume, but we figured that we had pretty completely replaced the rat's blood volume. In the few rats who survived - it was a difficult experiment - we were unable to show any kidney lesions or even increased proteinuria over the next few days. Therefore, it seems that one single large dose of serum from a nephrotic rat was not effective in doing the same thing as chronic dosage in your parabiotic rats appeared to do.

DR. PFEIFFER. *I agree completely and I think it is mainly a question of quantity,* that is, how much and how long these hypothetical autoantibodies are allowed to attack their parenchymatous tissue antigens in healthy individuals. Also I think that it might be possible to transfer such an inflammatory process only by means of transfusion, that is, without the parabiosis, if you provide a reliable transfer of large amounts of blood or serum from one animal to another; e.g., in rabbits this should be done by using this beautiful technique of drawing blood from the arteries of the ear, demonstrated so nicely by Dr. Lange. Then, large amounts of blood, serum or gamma globulin of diseased rabbits could be given to healthy rabbits.

In regard to such transfusion experiments in human nephrotics I should like to mention Dr. Poli in Milan, whom I visited some years ago. He transferred blood from patients with nephrosis to others with cancer, and two days later observed proteinuria.

If I remember right he published this observation in an Italian book [71].

To summarize, I think the parabiotic procedure is not necessary to induce nephritis, encephalitis or ophthalmitis in healthy individuals with the blood of diseased individuals *of the same species. Repeated transfusions of large quantities of blood or serum should* do it alone.

DR. GEORGE SCHREINER (Georgetown University Hospital): I wonder if anyone recalls the patient on the west coast who was cross-transfused? The child with nephrosis was connected in vivo-transfusion with his father and the father died.

There was clearing of edema through the father's kidneys; but, thereafter, the father developed an aplastic anemia which, I understand, was diagnosed as a sort of anaphylactic reaction of the bone marrow; and the father subsequently died.

DR. WALLACE McCRORY (Philadelphia, Pennsylvania): Would someone comment on what I find a little confusing. I believe it has been reported before - I am embarrassed that I do not remember by whom - that clamping a kidney and injecting sera prevented development of nephrotoxic disease in that kidney. How was the observation compatible with these results?

CHAIRMAN HEYMANN: I believe that Dr. McCrory refers to the work that we published some years ago, when the left renal pedicle was clamped for twenty-five minutes after the injection of anti-kidney serum [72]. Not all animals developed unilateral disease. As far as I remember, only 30 per cent of the rats developed nephrotic *renal disease on the non-clamped side only. This was established by histological* control in each instance, and Dr. McCrory is correct in finding this incompatible with the interpretation advanced by Dr. Pfeiffer for the results that he obtained in his parabiotic experiments. I believe that a different interpretation has to be considered, in that the parabiosis was established well within the half-life time for heteronephrotoxic antibodies, which is *about six weeks. Thus, it is possible that the still present antibodies* wandered from one animal to the other. If the parabiosis would have been established two or more months after the injection of anti-kidney sera, we would be hard up for an explanation.

DR. PFEIFFER: *We did experiments* [73] which were similar to yours, Dr. Heymann. *We repeated* the studies, originally published in 1939 by Sarre and Wirtz [74]

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[71] Poli, E., Fisiopatologia e clinica del Protido Plasma, Societa Editrice, Delfino-Milano, 1951.

[72] Heymann, W. and Hackel, D. B., Role of Kidney in Pathogenesis of Experimental Nephrotic Hyperlipemia in Rats. Proc. Soc. Exper. Biol. and Med., 89. 329, 1955.

[73] Pfeiffer, E. F., Die Bedeutung der tierexperimentellen Glomerulonephritis fuer das Verstaendnis der menschlichen Nephritispathognese, Habilitationsschrift, Frankfurt/Main, 1955.
Schoeffling, K., *Einseitige* Funktionsbehinderung einer Niere und experimentelle Glomerulonephritis, Verh. Dtsch. Ges. Allergieforschg. and Verh. Dtsch. Ges. Ophthalmologie (Joint meeting), 8-10 Sept. 1957, Heidelberg (in press).

[74] Sarre, H. and Wirtz, H, quoted in [73].

in rabbits after injection of nephrotoxic sera from ducks. We used rats which were given rabbit anti-rat nephrotoxic serum. However, we did not observe unilateral nephritis in rats. We observed the same intensity of glomerulonephritis on both sides histologically. In one out of seven animals, we found one degree less glomerulonephritis in the clamped kidney than in the kidney of the other side.

Furthermore, we studied the same problem by producing an experimental hydronephrosis on one side, because of a case report of Fahr in the German literature. In 1941, he described a case with unilateral hydronephrosis who died with glomerulonephritis, which was found only in the kidney which was not hydronephrotic [75].

In 1944, Reubi from Switzerland, reported that after unilateral experimental hydronephrosis in rabbits, the experimental glomerulonephritis following injection of nephrotoxic duck serum also was established only in the kidney without nephrohydrosis [76]. Therefore, we studied this problem in rats, but again, found no histological difference between the glomerulonephritic lesions in both kidneys [58].

DR. LANGE: Could it be that the difference here again is that we are dealing with two different diseases? Dr. Pfeiffer has transferred an antibody against kidney; and, until this antibody was completely absorbed onto the kidney which was still in the circulation, it would continue to damage the other kidney when the clamp was removed from it after twenty or thirty minutes.

The systems are different, too. With duck serum, you cause an immediate localization; and the animal forms its own antibodies against the antigen. When the clamp is removed, the previously clamped kidney has no localization, has no resilience; it is not immunized. When the antibodies come, they damage only one kidney, and not the one which has not been sensitized.

CHAIRMAN HEYMANN: I think there are a few things which a few of us have to get together and talk about privately! Dr. Goodman, Dr. Liu, and I, certainly should do so because we all did the same thing with different results. Once, we did compare our techniques, step by step, with Dr. Liu. The only difference that we could find was that Dr. Liu used lyophilized antigen, while our antigen solutions just had been kept frozen. Thus we repeated several sera after having lyophilized our antigen without obtaining different results. There possibly are other factors involved.

I will have to have another martini with Dr. Pfeiffer to talk things over.

If there are no other questions, the meeting is adjourned.

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[75] Fahr, G., quoted in [73].
[76] Reubi, quoted in [73].

II. LIPOPROTEIN METABOLISM

The Conference reconvened at nine-fifteen a.m. Dr. Baxter presiding.

CHAIRMAN BAXTER: We have a long program this morning; I think we had better get started. Dr. David Gitlin is going to start off with discussion of lipoprotein metabolism.

A. Lipoprotein Metabolism

DR. GITLIN: Thank you Dr. Baxter.

This morning, I have been asked to discuss some of our work on lipoproteins. The individuals primarily concerned were Dr. Dave Cornwell, Mrs. Doris Nagasato, Dr. J. L. Oncley, Dr. Walter Hughes, Dr. Charles A. Janeway, and Dr. Paul Gross.

The size of our group is a little out of proportion with the results. (Laughter)

The lipoproteins, labelled with I^{131}, were isolated in the conventional manner of flotation in solutions of differing specific densities by means of ultracentrifugation. The labelled isolated lipoproteins were studied immunochemically and electrophoretically, as well as ultracentrifugally.

With regard to ultracentrifugal analysis, after labelling, over 90 per cent of the labelled protein could be recovered in the analogous fraction when it was added either to unlabelled serum or to unlabelled carrier protein. With electrophoresis, we obtained the expected patterns: In starch electrophoresis the labelled lipoproteins migrated with the analogous, unlabelled fraction.

The chylomicra labelled very poorly or not at all with iodine, because of their very low protein content.

In any event, we eliminated the chylomicra from consideration in this study, by centrifugation at 25,000 G. for about thirty minutes before we analyzed any samples. By this procedure, we did not lose any label.

The normal individuals we studied were adult volunteers, and the patients were children. We could find no difference between the turnover of the lipoproteins in a child who had recovered from the nephrotic syndrome and the turnover in adults.

In normal adults, Sf 3-9 lipoproteins disappeared with a half-life of approximately three days.

The half-life or the turnover half-time of the iodinated Sf 3-9 lipoprotein was a little bit faster, not very much, in nephrotic children than in the child who had recovered from the nephrotic syndrome, or in the normal adult.

In these nephrotic children, the cholesterol attributable to the Sf 3-9 lipoprotein was either normal or somewhat decreased. This would indicate that the metabolism of the Sf 3-9 lipoproteins was comparatively normal in these particular children; the rate of synthesis was normal; and the turnover half-time was increased only slightly.

After intravenous injection of labelled Sf 10-200 lipoproteins in normal persons, increasing amounts of label became associated with the 3-9 lipoproteins. Apparently, a conversion was taking place, the Sf 10-200 protein moiety becoming Sf 3-9 protein moiety. The loss of radioactivity from Sf 10-200 lipoproteins in the nephrotic individuals was considerably slower than occurred in the normal individuals.

Thus the fractional rate of conversion of the Sf 10-200 lipoproteins in the nephrotic child is quite decreased compared to normal adults.

After a series of calculations, partially based upon several assumptions which may not be true entirely, we decided that the following scheme represents the metabolism of Beta-lipoproteins in normal and nephrotic individuals.

In the normal individual, there is synthesis of low-density beta lipoproteins, Sf 10-200. There may be some synthesis of the high-density beta lipoproteins as well, we do not have evidence either for or against this. The individual low-density lipoprotein molecule either may be converted to a higher-density beta lipoprotein, Sf 3-9 or may be catabolized. The Sf 3-9 molecule goes on to be catabolized as well. The major pathway in normal persons is through the high-density beta lipoproteins. In this particular scheme, the work of a number of investigators indicates that lipids are released during the conversion process, and that, if they accumulate, there is an inhibition of conversion. The lipids are removed by combination with albumin. So, for all practical purposes in the normal individual, there is no inhibition of this conversion. In the nephrotic individual, the major pathway seems to be to the synthesis of low-density lipoproteins. For some reason, the conversion of low-density to high-density lipoproteins is inhibited. As a consequence, there may be an accumulation of Sf 10-200 lipoproteins. The major pathway for removal of Sf 10-200 lipoproteins in the nephrotic child is through catabolism; some go through the pathway of conversion and then become catabolized.

Based on the correlation which a number of people have found between hypoalbuminemia and hyperlipemia or hypercholesteremia, we assumed that the albumin was involved. Rosenman and others have indicated in rats that, if one infuses albumin, the accumulation of lipids is prevented.

We find now that this is not the entire picture. By some odd set of circumstances, in each of the five or six patients we studied, the hypercholesteremia was attributable to an increase in the low-density lipoproteins, Sf 10-200. When we studied this process a bit more, we began to find other individuals, as Dr. Baxter found, who had only a slight increase, a three-fold increase, in the Sf 10-200 lipoproteins. In these particular

individuals, the hypercholesteremia was attributable mainly to an accumulation of Sf 3-9 lipoproteins.

We gave heparin to a number of nephrotic children. Some patients would clear the Sf 10-200 lipoproteins very rapidly; in others, converson would not take place. We began to realize that there was something more than just albumin involved; and we came to the conclusion that the other material which is involved is lipoprotein lipase or its equivalent; namely, that, in those nephrotic individuals in whom there is an accumulation of the Sf 10-200 lipoproteins, lipoprotein lipase was deficient. If we give other individuals heparin, they remove what low-density lipoproteins they have accumulated, and these go, apparently, into the high-density fraction. In those individuals who have an increase in the high-density beta lipoproteins, they apparently can clear with heparin, in addition to which the fractional rate of turnover is apparently normal. This would indicate that there is an increase in the synthesis of the high-density beta lipoproteins.

Whether this increase in synthesis results from an increase in conversion, or whether this pathway exists at all, we cannot say; but our present feeling in the matter is that there may be a variety of defects in the nephrotic syndrome, that there is an increase in the synthesis of the low-density lipoproteins, and a variable degree of inhibition of conversion. If this block is overcome, or if it did not exist in the first place, accumulation of lipids would occur in the Sf 3-9 lipoproteins.

I think Dr. Bally will give further evidence that albumin is not the only factor in inhibition of the conversion process.

DR. PETER BALLY: I will talk about it at eleven o'clock.

CHAIRMAN BAXTER: I think we should have a limited discussion of this paper now. Some of the other papers will bear on the same subject. Perhaps we can save some of the general discussion until after at least the first three papers.

I would like to ask Dr. Gitlin, just to begin with, why he thinks lipoprotein lipase is deficient, and just where the deficiency is. Then perhaps Dr. Korn or Dr. Rodbell will comment on this.

DR. GITLIN: Whether it is lipoprotein lipase we do not know. We know that in the particular individuals we studied they do not clear with heparin; that is to say, there is no conversion, there is no loss of radioactivity from the Sf 10-200 into the Sf 3-9 fraction.

In those individuals whose hypercholesterolemia is due to a high concentration of high-density beta lipoproteins (that is, Sf 3-9), when we give labelled Sf 10-200 lipoproteins, then give heparin, conversion of Sf 10-200 to Sf 3-9 lipoproteins may occur, and a drop in cholesterol and in the Sf 10-200 lipoproteins, which one cannot get in those individuals in whom the major cause of hypercholesterolemia is due to accumulation of the Sf 10-200 lipoproteins. In those children who have hypercholesterolemia due to accumulation of Sf 3-9 lipoproteins, the turnover of the Sf 10-200 is relatively normal.

So, if we can equate heparin with some clearing factor I suppose the best thing to do would be to call it simply some clearing factor rather than lipoprotein lipase; but it is obvious that it has to be lipoprotein lipase by definition.

DR. WALLACE McCRORY (Children's Hospital, Philadelphia, Pennsylvania): In calculating your turnover rate, is it not true that the fact that there is an increased plasma pool of lipoprotein can account in part for the lower rate of turnover? With five times the amount of lipoprotein, there would be one-fifth the chance that the tagged molecule would be taken in comparison to the normal person with a smaller pool, even though the turnover rate was the same. Could you clear this up?

DR. GITLIN. One of the big difficulties in trying to make any calculations with regard to the children with nephrotic syndrome is the question of pool size.

In any event the fractional rate of catabolism is a first order of reaction and not dependent upon pool size as a first approximation.

We have estimated the pool size of these lipoproteins in nephrotic children by tapping the extravascular fluids; there was a shift in the distribution so that most of the pool was in the plasma. The change was on the order of tenfold.

DR. RAPOPORT: How does this compare to familial hyperlipemia where everything else is normal? In general, there is a correlation between hyperlipeima and low plasma albumin, but in this familial disorder, albumin and other proteins are normal, yet there is hyperlipemia. My guess is, it resembles the hyperlipemia of the normal patient.

DR. GITLIN: It resembles that metabolically as well.

DR. RAPOPORT: It seems to me you would get a better standard of reference if you compared familial hyperlipemia to the normal and then to the nephrotic, where an awful lot of things are going on all at once.

DR. GITLIN. Yes. We have done some patients with this particular disease, and found that, in these particular individuals, when compared to normal persons, the situation is pretty much like that found in the nephrotic syndrome; namely, there is a decrease in the rate of conversion of very low-density to high-density beta lipoproteins. The people at the National Institutes of Health, as a matter of fact, have shown that this is due to a decrease in lipoprotein lipase.

DR. RAPOPORT: Demonstrable or assumed?

DR. GITLIN. Assumed. It is a very difficult thing to measure.

DR. ROBERT GORDON (National Heart Institute): Dave, there is one slide which I would like to see again for a minute, one about kinetics of lipoprotein turnover.

You mentioned that the lower curve shows a much more rapid degradation of Sf 3-9.

DR. GITLIN: No, I did not mean degradation.

DR. GORDON: I wondered if this was not the point, that the limiting slope on these two curves is very parallel - if there is a difference, it is a very slight one - and the lower curve looks as though the tracer is distributed into some functional, metabolic or other compartment which is quite a bit larger than normal. The upper curve is the nephrotic patient; and the injected tracer is the Sf 10-200. I wonder if this does not merely imply that the ratio of 20-100 to 3-9 is lower in the normal person than in the nephrotic; so, if you do get this conversion of isotopic 20-100 into 3-9, it goes on to a much larger extent in the normal; but, once these two pools are equilibrated, it looks as though the combined pool now is decaying similarly.

This would seem to imply that this conversion of 20-100 into 3-9 would be a reversible process. If it is somewhat reversible having the two compartments behaving functionally as one after they have equilibrated, so that we get this parallel slope, it would seem reasonable; whereas, if the conversion is strictly a one-way affair, I do not understand this parallelism.

DR. GITLIN: They are artificially parallel.

If we give Sf 3-9 lipoproteins, we find none of it coming back into Sf 10-200. However, you could argue that, in the iodination procedure, we blocked certain groups which are necessary for reversal.

These curves are not really parallel. These are plasma-disappearance curves-and the plasma-disappearance curves in the normal individual in this particular instance do not reflect the behavior of the whole pool but only newly synthesized molecules just as in the case of the plasma-disappearance curve of albumin in a nephrotic individual.

This curve represents a minimum of three components: disappearance in the extravascular system, conversion, and catabolism. What happens in this particular instance is that there is a very rapid disappearance from the vascular system. disappearance by diffusion and by conversion. Under these circumstances, the specific activity in the extravascular pool is elevated about threefold. The higher specific activity in the extravascular compartment then diffuses back into the plasma compartment, resulting in a decreased slope of plasma disappearance. The situation is not quite the same in the nephrotic child.

If we say that the Sf 10-200 group is heterogenous with respect to conversion and some of it is not converted, the problem gets even more complex.

DR. GORDON: Is it like that; or it is just parallel?

DR. GITLIN: No, because this does not operate in nephrosis, because most of the lipoprotein in the nephrotic is in the plasma compartment, and very little of it is present in the extravascular compartment. This is not so in the normal, which is why these calculations are so difficult and so shaky from the very beginning.

We can only say very definitely that the Sf 3-9 lipoproteins are catabolized in a normal manner; and, in certain nephrotic individuals, there is an increase in synthesis. In those individuals who have hyperlipemia due to the low-density lipoproteins, the synthesis of Sf 3-9 lipoproteins is normal, no matter by which pathway.

I have not brought in the alpha lipoproteins; but the alpha lipoproteins are very simple to go through. All that happens with the alpha lipoproteins is that there is an increase in the rate of degradation; and the rate of synthesis is normal. The result is a lower concentration of alpha lipoproteins in nephrosis.

CHAIRMAN BAXTER: Dave, may we go ahead, and continue the discussion as we go along?

(Dr. Gitlin assumed the chair.)

CHAIRMAN GITLIN: Well, Dr. Baxter, you are the next one on the program. Dr. Baxter will discuss lipoprotein patterns and the effects of albumin infusions in nephrotics.

B. Lipoprotein Patterns and Effects of Albumin Infusions in Nephrosis

DR. JAMES H. BAXTER (National Heart Institute, National Institutes of Health, Bethesda, Maryland): I want to discuss two related topics which have already been touched on by Dr. Gitlin. One is the abnormal lipoprotein patterns which occur in nephrosis. The other is the effect of albumin infusions on the lipoproteins, or the question, perhaps, of what is the role of the hypoalbuminemia in the causation of the abnormal lipoprotein patterns in nephrosis. Various portions of the studies which I will describe have been done with the aid of Drs. Howard Goodman, Joseph Bragdon, Richard Havel and Robert Gordon.

After first carrying out lipid determinations on aliquots of the whole sera, which were obtained under uniform fasting conditions, we have separated the lipoproteins of the sera from nephrotic patients into three fractions by ultracentrifugation at appropriate densities[1]. First the serum is made to a density of 1.019 with solutions of NaCl and KBr, and the lipoproteins which rise to the top on centrifugation at this density are separated. This fraction contains the very low density ($D < 1.019$, $Sf > 10$) lipoproteins, including the chylomicrons. The infranate is then brought to a density of 1.063 and the low density lipoproteins (D1.019 - 1.063, Sf 0-10) are separated at the top. The infranate from this second centrifugation is made to density 1.21 and again subjected to centrifugation (at 100,000 G for 48 hrs.), followed by separation of the material in the top of the tube from the infranate. The supernate contains the high density lipoproteins which migrate with the alpha-1 globulins in starch electrophoresis and the infranatant contains the remaining serum proteins and several minor components. Lipid and protein analysis are then carried out on each of the separated fractions.

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[1] Havel, R. J., Eder, H. A., and Bragdon, J. H., The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest., 34. 1345, 1955.

Before discussing the lipoprotein abnormalities in nephrosis, I would like to point out the practical value of ratios of total cholesterol to triglycerides (TC/TG) in whole serum for predicting lipoprotein composition of the serum. The lipoproteins of D<1.019 have a high content of triglycerides with a TC/TG ratio which normally is about 0.3[2]. An increase in this fraction, without other alterations, would decrease the TC/TG ratio of whole serum which is normally about 1.8. On the other hand, the TC/TG ratio of the lipoproteins of the D 1.019-1.063 fraction is about 3, and an increase in this fraction alone would increase the TC/TG ratio of serum. A simultaneous increase in both of these fractions might have little effect on the TC/TG ratio of the serum. Alterations in the quantity of the high density (D 1.063-1.21) lipoprotein fraction, which usually are not great, would not significantly change the TC/TG ratio of serum. It is evident therefore that the lipoprotein composition of serum can be fairly accurately predicted from the lipid analyses on whole serum, provided that the composition of the individual lipoprotein fractions remains normal. These ratios have been fairly reliable in our nephrotic patients, though some alterations in composition of the fractions do occur. If the TC/TG ratio of the whole serum is less than 1, the predominant elevation is in the very low density (D<1.019) fraction. If the TC/TG ratio is greater than 2, the elevation is chiefly in the low density (D 1.019-1.063) fraction. If the TC/TG ratio is between 1 and 2, neither of these fractions is greatly elevated or else both fractions are elevated. Since increase in the very low density (D<1.019) fraction with high content of triglycerides are responsible for the lactescence of serum, the lipoprotein composition of serum can be predicted with reasonable accuracy from the appearance of the serum and the cholesterol level. A cholesterol determination alone is inadequate.

The typical serum in nephrosis has usually been described as lactescent or lipemic. However, we have seen nephrotic patients with considerably elevated cholesterol levels whose sera were clear[3]. Data from representative patients are shown in Table 9. It is evident that either or both of the fractions D<1.019 and D 1.019-1.063 may be elevated in nephrosis. The first line of data in Table 9 are normal values from young patients studied by Havel et al. The other data are from individual nephrotic patients whose initials and ages are given in the left-hand column. Analyses on the whole sera are given in the central portion of the table, and total cholesterol values on the three lipoprotein fractions (as indices of the quantities of the fractions) are given in the right-hand portion of the chart. It should be pointed out that no one constituent is an exact index of the fractions because the composition of the fractions is often abnormal in nephrosis. For example, the ratio of cholesterol to triglycerides is often greater than 0.3 in the D<1.019 fraction and the TC/PL ratio of the fractions may be abnormal.

In the first four cases in Table 9, the D<1.019 fraction alone was elevated. It may be noted that the serum TC/TG ratios were well below 1, and the sera were very creamy. In several patients with this pattern, the D<1.019 material has been further fractionated by centrifuging it at a density of 1.006 and the major portion has been found to rise (D<1.006). In the second group of patients in Table 9, both the D<1.019

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[2] Bragdon, J. H., Havel, R. J., and Boyle, E., Human Lipoproteins. I. Chemical composition of four fractions. J. Lab. and Clin. Med., 48: 36, 1956.

[3] Baxter, J. H., Goodman, H. C., and Havel, R. J., Hyperlipidemia of nephrosis. J. Clin. Invest., 36: 873 (abstract), 1957.

TABLE 9

LIPIDS AND LIPOPROTEIN FRACTIONS IN SERA OF PATIENTS WITH NEPHROSIS

	Serum							Lipoprotein fraction (density)		
	TC	FC	PL	TG	TC/TG	Appearance	Albumin	1.019 TC	1.019-1.063 TC	1.063-1.21 TC
	mg/100 ml.						gm/100 ml.	mg/100 ml.		
Normal	180	45	225	100	1.8	clear	4.0	25	100	50
SS*-29	1522	435	942	3210	0.5	very creamy	0.8	1247	98	46
LG-22	1234	349	785	2440	0.5		0.6	1023	78	21
WT-10	715	235	636	2387	0.3		0.5	624	63	19
JH-2½	677	200	620	3920	0.2		0.6	609	52	16
OH-41	588	149	497	570	1.0	slightly creamy	1.1	326	229	46
RS-3½	682	154	562	390	1.7		0.6	234	333	54
FF-30	471	125	419	400	1.2		0.8	208	201	47
EW-50	857	251	691	480	1.8		0.9	210	604	57
SS*-31	379	91	399	197	1.9	clear	2.5	23	219	68
AR-4	586	124	479	120	4.9		2.0	60	382	85
DP-3	703	166	514	174	4.0		0.9	48	541	97
MH-5	678	181	499	169	4.0		1.2	64	520	49

TC = total cholesterol   PL = phospholipids
FC = free cholesterol   TG = triglycerides

and the D 1.019-1.063 fractions were moderately elevated. In the third group of patients in Table 9, the D<1.019 fraction was approximately normal (note that triglycerides were only slightly elevated and sera were clear) but the D 1.019-1.063 fraction was considerably elevated (as was serum cholesterol and phospholipid). Probably there is a continuous spectrum of lipoprotein patterns in nephrosis rather than a clear-cut division into three groups. Some patients have changed from one pattern to another under observation. In general, the first pattern described has been associated with the lowest levels of serum albumin and the more severe forms of the disease symptomatically, while the third pattern has often occurred in patients with higher serum albumin levels and less marked edema and ascites. The lipoprotein alterations in the three groups of patients are summarized in Table 10.

TABLE 10

ALTERATIONS OF LIPOPROTEIN FRACTIONS IN NEPHROSIS (SUMMARY)

	Fraction (density)		
	1.019	1.019-1.063	1.063-1.21
Group 1	++++	0 to -	-
Group 2	++	++	0
Group 3	+ to 0	+++	+ to 0

CHAIRMAN GITLIN: May I ask, Jim - I remember, years ago, Eder* showed that the concentration of phospholipids does affect the lactescence or nonlactescence of the lipemic serum.

I do not know whether the phospholipids have any effect on the distribution of low and high density lipoproteins.

DR. BAXTER: Of course it was not realized until fairly recently that the lipids of serum are combined with one another and with protein in the form of a series of large lipoprotein molecules. It is true that the sera which are very creamy have a high triglyceride content and a much lower cholesterol and phospholipid content, so that it might be said that the sera with very low phospholipid in relation to total lipid are creamy. However, we have not found the ratios of cholesterol to phospholipid in lactescent sera to be consistently different from those in clear sera, and I think this is at variance with the old observation to which you refer. If the very low density lipoproteins are significantly elevated, the serum will appear lactescent; if only the lipoproteins of higher density are elevated, the serum remains clear.

CHAIRMAN GITLIN: I think it was Eder who found that, after the addition of phospholipids to a lactescent hyperlipemic serum, he could clear the serum.

DR. BAXTER: I remember something about that; but I do not remember the details. This would be an artificial situation.

*Eder, H. A., Third Annual Conference on The Nephrotic Syndrome, 1951.

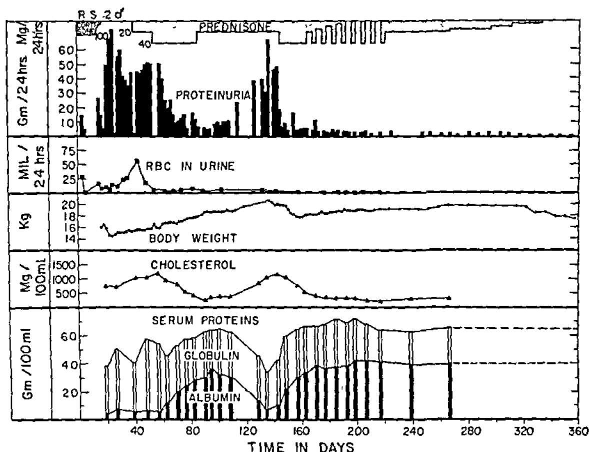


Fig. 36 Course of patient with nephrosis, showing relationship between levels of serum albumin and cholesterol.

DR. JOHN A. JAMES (Dallas, Texas): Dr. Baxter, do you feel that the lipi pattern in any one individual can vary from time to time, or do the patterns defin different types of disease?

DR. BAXTER: No, I do not think they are different types of disease, because w have seen patients change from one pattern to another. For example, patient SS (Tabl 9) was admitted with a very low level of serum albumin and severe manifestations c nephrosis and was found to have the first lipoprotein pattern which I described, wit creamy serum. He had a complete remission of the disease induced by steroid therap but later had a symptomatically mild relapse associated with the third lipoprotei pattern, with elevated cholesterol but clear serum.

DR. RAPOPORT: What happens in a patient whose serum you clear with heparin

DR. BAXTER: We have not studied that in nephrotic patients.

I would like to go on with the second topic, the effects of albumin infusions on th abnormal lipoproteins.

It has of course been recognized for a long time that there is some sort of a inverse correlation between serum albumin level and cholesterol level in nephrosis Figure 36 illustrates this relationship in one of our patients treated with steroids As the serum albumin level went up the cholesterol level fell, and so did the level o the other lipids. This does not prove that the lipoproteins are directly influenced b the level of albumin. It is conceivable that as a remission of the disease occurs, albumin and the lipoproteins independently change toward normal. On the other hand, there is absolutely no reason to assume (as has been done) that there would invariably be a specific mathematical relationship between cholesterol and albumin levels, if the lipoproteins are influenced by albumin level. In the first place, as was evident from the first part of this discussion, a cholesterol determination gives only limited information about the composition and quantity of the lipoproteins present. Furthermore, it is obvious that the lipoproteins are influenced by a number of factors.

In order to determine what effect serum albumin level has on the abnormal lipoprotein pattern in nephrosis, we have attempted to change the serum albumin level without changing the disease itself by giving repeated albumin infusions. In order to obtain definitive results it is necessary to have an adequate control period and a reasonably stable state and to give large quantities of albumin for several weeks; otherwise interpretation of the results is complicated by spontaneous variations, hemodilution and other factors. We have carried out studies with albumin infusions in 7 patients but for various reasons only 3 or 4 have been reasonably satisfactory.

Figure 37 shows the effects of albumin infusions over a period of 4 weeks in a 14 year old girl. This patient's serum albumin level was not as low as is often seen and the lipoprotein elevation was largely, but probably not entirely, in the D 1.019 - 1.063 (Sf 0-10) fraction as indicated by the TC/TG ratio near 2 and clear serum. As the serum albumin level increased, it can be seen that there was an impressive fall in total cholesterol to near normal levels, and also a fall in phospholipids. Changes in triglycerides, which were never greatly elevated and which often behave in a somewhat

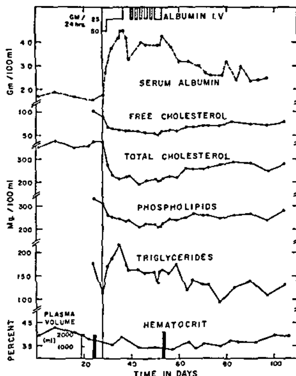


Fig. 37 Effects of albumin infusions on serum lipids in a nephrotic patient with clear serum

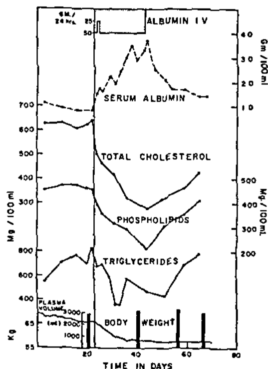


Fig. 38 Effects of albumin infusions on serum lipids in a nephrotic patient

erratic manner, are difficult to interpret. It can be concluded that the D 1.019 - 1.063 lipoprotein fraction in this patient decreased to near normal levels as a result of albumin infusions, and that the decrease was not a result of hemodilution. This experiment left something to be desired, in that the level of serum albumin did not return to pre-treatment level.

The effects of daily infusions of 50 gm. of albumin for 3 weeks in a 36 year old man are shown in Figure 38. In this patient the serum albumin level was 0.8 gm. % and, in contrast to the first case, the major lipoprotein abnormality (at the time albumin infusions were begun) was an elevation of the very low density (D< 1019, Sf>10) lipoprotein fraction. The triglyceride elevation was greater than that of cholesterol, and the serum was moderately milky. Again a precipitous fall in cholesterol and also in phospholipids occurred as the serum albumin level rose, and in this case there was also a definite fall in triglycerides. The lipid levels again rose as the serum albumin returned to the pretreatment level. During the albumin infusions there was a complete loss of edema, and a slight increase in plasma volume.

The changes in lipid components of the various lipoprotein fractions in the second albumin experiment are shown in Figure 39. It will be noted that there was a considerable decrease in the very low density (D<1.019) fraction which is the one which was elevated. It is also evident that the composition of the very low density fraction was abnormal.

These two examples serve to illustrate that infusions of albumin which significantly increase the serum albumin level may result in a decrease in the abnormal lipoproteins, whether the elevation is in the D 1.019 - 1.063 fraction or the D<1.019 fraction. The lipoproteins in most cases did not become completely normal, but neither was the serum albumin maintained at completely normal levels. I certainly do not want to say that the only cause of the lipoprotein abnormality in nephrosis is the low serum albumin level; in fact, we have made some observations which suggest that albumin may not be the only factor. For example, we have seen considerable decreases in the very low density lipoproteins occur early in remissions, before there has been much increase in serum albumin levels.

Albumin does have a definite effect on the lipoproteins, and I do not know what the mechanism is. It may be that the stimulus which results in the lipoprotein abnormalities is a result of the decreased oncotic pressure of the serum. If this is so, dextran infusions should bring about a change toward normal. Another possibility which suggests itself is related to the transport of unesterified fatty acids which Dr. Gordon will discuss later. Since unesterified fatty acids are transported bound to albumin[4], it is conceivable that an abnormality in this mechanism when serum albumin is abnormally low might somehow result in the lipoprotein abnormality. Figure 40, about which Dr. Gordon will have more to say, shows the relationship between molar concentrations of unesterified fatty acids and albumin in 3 nephrotic patients before, during and after treatment with albumin and prednisone. The case at the top is the first case treated with albumin in which lipoproteins of D 1.019 - 1.063 were elevated.

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[4] Gordon, R. S., Jr., and Cherkes, A., Unesterified fatty acids in human blood plasma. J. Clin. Invest., 35: 206, 1956.

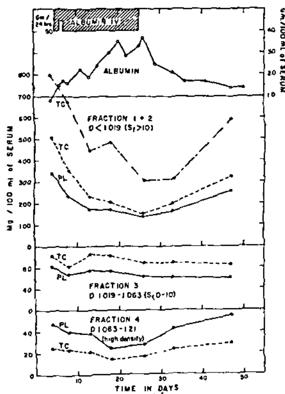


Fig. 39 Effects of albumin infusions on lipid components of individual lipoprotein fractions (Patient same as in Fig. 38.)

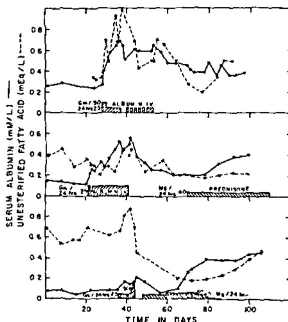


Fig. 40 Relationships between molar concentrations of albumin and unesterified fatty acids in 3 nephrotic patients under various conditions.

The other two patients initially had lactescent sera with considerable elevation of the $D<1.019$ lipoprotein fraction. It is interesting that only during periods of elevation of the very low density lipoprotein fraction was the molar ratio of unesterified fatty acids to albumin consistently above 2.

CHAIRMAN GITLIN: Are there any questions anyone would like to ask at this time?

DR. BALLY: I would like to ask Dr. Baxter something about the level of cholesterol and its relation to cholesterol intake, which he had mentioned to me previously. If there is a higher intake, does the cholesterol become elevated proportionately to the triglycerides?

DR. BAXTER: We really have not studied that. I cannot answer that question, except to say that we have seen some instances, in treating nephrotic patients, where for some reason their food intake dropped off rather markedly, and the cholesterols also dropped. When their appetite picked up and they started eating again, the cholesterol levels rose. Of course, there is much more information about this point than we have; we have felt that there is some relationship.

DR. METCOFF: Jim, is there any relationship between the occurrence of the $Sf>10$ fraction, and the appearance of xanthomata? Because it is curious that, in nephrotic children at least, xanthomata frequently tend to occur when there are very high cholesterol levels, around 900 or higher.

DR. BAXTER: I have not seen xanthomata in these patients.

DR. RAPOPORT: Jack, the appearance of xanthomata is conditioned purely by something traumatic happening to the skin. If you do a paracentesis, you will get xanthoma at the site. There are two very dramatic ones with children. They have marked prickly heat and, I am sure, localization conditioned by some irritated focus in the skin, singularly or in multiple. My guess is it has nothing to do with the level of the blood cholesterol itself.

CHAIRMAN GITLIN: Dr. Baxter, since I am Chairman of this particular session, I shall exert my prerogative and abandon your procedure in order to have a full discussion after each paper, if you do not mind.

DR. BARNETT: I would like to ask, Jim, whether you have observed any patients who appear to be in complete remission in terms of plasma proteins and rate of protein excretion, but who have elevated concentrations of serum cholesterol. If so, have you studied them in this way?

DR. BAXTER: I do not think I can answer that completely. Of course, some of the patients might have elevated cholesterols for other reasons, I mean aside from nephrosis. But certainly, in general, our patients in whom the proteinuria has cleared up, and the serum albumin has risen to a completely normal level, I think the lipids have become completely normal, although sometimes it has taken quite a while.

DR. BENJAMIN KRAMER (The Jewish Hospital of Brooklyn): I have seen patients who, under treatment, had no proteinuria, but still had a moderately elevated cholesterol; and it took quite some time before the cholesterol also returned to normal.

CHAIRMAN GITLIN: We have all seen patients like that; but do you know what the albumin levels were in these particular patients, Dr. Kramer?

DR. KRAMER: They were quite normal.

CHAIRMAN GITLIN: It has been my experience that it takes quite a little while for the albumin to return to normal levels. But this may be a rare phenomenon.

Is it conceivable, Dr. Baxter, or does it conflict with your ideas on the subject, that the infused albumin had nothing whatever to do with the removal of the cholesterol but, instead, inhibited synthesis of the lipoproteins?

DR. BAXTER: I have no information on that point.

DR. PFEIFFER: I have no experience with children; but we have always wondered what occurred when a nephrotic changed to the stage of so-called nephrosclerosis. The same changes you have shown occurred, the cholesterol went down, and the blood proteins went up. We had three or four such cases. What is your experience?

DR. BAXTER: I am not sure I understood the question. You say these patients no longer had proteinuria.

DR. PFEIFFER: If there was 12-14 per cent proteinuria initially there was merely 0.4 per cent in the final stage, and the BUN became high.

CHAIRMAN GITLIN: Do you know the serum albumin levels on those cases?

DR. PFEIFFER: Yes; they were high.

DR. BAXTER: We have followed very few patients with chronic nephritis. I think, in nephrotic patients we have followed, as I said, when albuminuria clears up, serum albumin rises; lipids come down.

I will not go into this; but Dr. Gitlin has been interested, I know, in the quantitative relationship in the steady state of albumin in the urine to the serum albumin levels. We have followed this relationship in a number of patients. While we have seen some patients, as Dr. Gitlin has, where the serum albumin level seemed to be lower than would be predicted by the loss of albumin in the urine, in general that has not been the case. When the albuminuria cleared up, the serum albumin became normal and the lipids became normal.

DR. DONALD GRIBETZ (Department of Pediatrics, Mt. Sinai Hospital, New York): Have you studied any hypercholesterolemia in children in which the albumin could be normal, like, for example, hypothyroidism?

DR. BAXTER: I have not studied any; but some of the people present here have. Dr. Fredrickson has studied patients with various types of hypercholesterolemia; so has Dr. Gordon. Perhaps they would like to comment on this.

DR. GRIBETZ: I am interested in both the type of lipid and the changes which occur with remissions and exacerbations, with thyroid therapy or without it.

DR. DON FREDRICKSON (National Institutes of Health): Most hypothyroid children and adults have an increase in the so-called Sf 3-8 fraction. Usually, one does not see creamy serum, but it does occur.

After therapy, the cholesterol level usually follows the increase in metabolism gained by therapy.

DR. GRIBETZ: But this has nothing to do with changes in the protein level.

DR. FREDRICKSON: Not in the albumin level; no.

DR. WALLACE McCRORY: I would like to ask Dr. Baxter if he could say anything about any noticed depreciation in renal function in these patients. In one experience we had in giving albumin for one month to a patient, a marked decrease in renal function was observed. Their proteinuria, I assume, was markedly increased during the periods of albumin infusion.

DR. BAXTER: Yes; the proteinuria was markedly increased, of course, through infusions. In fact, some patients whom I did not show lost the albumin almost as fast as it was given. There was no change in renal function as measured by creatinine, and inulin clearances in some cases, in these patients.

I said they changed somewhat. We had two patients who seemed to be definitely better after the albumin infusions; that is, the serum albumin and the proteinuria stabilized at somewhat more favorable levels than prior to the albumin therapy.

DR. LANGE: Did you ever find that any of the protein actually was retained in the body after infusion?

DR. BAXTER: We have not done balance studies. I think it is obvious that some of the protein which we gave was retained for a while in these patients. I am not sure I understand the question.

DR. LANGE: Within, let's say, within 48 or 72 hours, was not all the albumin you had given excreted into the urine?

DR. BAXTER: Well, of course, it varied tremendously from one patient to another; I think that, in most cases not all of it was lost within 48 hours. But, in many patients a very considerable part of it was excreted within 48 hours, or even less.

CHAIRMAN GITLIN: It is pretty well known that with administration of albumin, anywhere from 50 to 75 per cent of it will be recovered eventually. Let's not forget

that some of it does remain in the body; also, a lot of it is being metabolized during the period of time it is present in the body, therefore you could not possibly get all of it back.

DR. METCOFF: And, of course, it varies from time to time, depending upon the status and stage of response of the individual patient.

I am sorry that Dr. Janeway is not here, because Drs. Janeway, Hutchins, and Barness, in the period from 1943 to about 1946, gave literally hundreds of infusions of albumin to nephrotic children, and made many observations, most of which, unfortunately, have not been reported. They found that there was a positive balance if albumin was infused after diuresis had begun, for example; while there would be an augmented loss of albumin during the initial therapy of an edematous child.

Most of the children who were so treated only had temporary remissions, a very few had more permanent ones. Most of the children recurred.

I believe they observed no significant change in gross renal function in association with the administration of albumin; but I do not know about glomerular filtration specifically, because only urea clearances were done at that time.

DR. RAPOPORT: Dr. McCrory mentioned a patient who, after a month's daily infusions of 5 g. of albumin, lost about 80 per cent of her glomerular filtration rate, and some years ago, I asked Dr. Janeway whether this had been his experience. I do not remember that he answered this directly; but he said yes, this could happen; that up to a certain date, about 1946, there was a good bit of mercurial preservative put in the concentrated albumin, and this included almost all of the infusions they gave.

CHAIRMAN GITLIN: Sodium cacodylate or tryptophane and cacodylate were used, as far as I remember.

DR. RAPOPORT: Giving a dose of merthiolate day after day may have modified some of the results obtained.

DR. METCOFF: I recall, rather vaguely, that George Cotzias at the Rockefeller Institite in the mid-forties, published some data on glomerular or tubular obstruction due to the administration of albumin, if I am not mistaken.

DR. BAXTER: I think you are referring to something I did with Cotzias, actually. (Laughter)

There was no evidence of damage from albumin. The kidneys did become markedly enlarged after a time, an enlargement in the tubules; but no evidence of damage, really. With gelatin, there was some.

DR. BARNETT: Jim, were you there when Eder, Lauson and Chinnard were giving large amounts of albumin?

DR. BAXTER: Yes.

DR. BARNETT: Wasn't it true that they had the impression also that in some children given large amounts of albumin, there may have been an acceleration in the rate at which some of those children lost kidney function?

DR. BAXTER: That possibility certainly was discussed, but I did not think they concluded that.

DR. HARRIET G. GUILD (Johns Hopkins Hospital, Baltimore): As evidence that the intravenous administration of serum albumin is not only not harmful but also beneficial and, at times, even life-prolonging, I should like to refer to a nephrotic child of the pre-cortisone era, to whom daily infusions were given for a period of eighteen months.

This child, then aged three years, 1½ years after the onset of nephrosis from which she had never had a spontaneous remission, was showing rapidly increasing impairment of kidney function when treatment with albumin was started. The non-protein-nitrogen was 90 mg.%, there was electrolye distortion in the direction of acidosis, and oliguria with urine volume amounting to not more than 200 cc. a day. In addition to special measures directed toward correction of the acidosis and other more general measures, daily injections of concentrated albumin, 40-60 cc. (10-15 gm.), were begun with a resultant increase in urinary output, decrease in non-protein-nitrogen, and stabilization of edema at a minimal level such that the child was at all times active and comfortable. With this her appetite improved sufficiently to make possible the maintenance of a good state of nutrition. Whenever the albumin was omitted for a day or two, as a test of its effectiveness, the urine volume fell, edema increased and the non-protein-nitrogen promptly rose. With resumption of therapy, there was a return of non-protein-nitrogen and urine volume to satisfactory levels which were maintained as long as treatment was not interrupted.

At no time, on this regime, were untoward reactions observed except occasionally when the albumin was injected too rapidly. At such times, a splotchy, erythematous rash appeared on the skin and there was increased intestinal peristalsis, evidenced by audible gurgling, which was followed by sudden vomiting. It was determined that these symptoms were related solely to the speed of injection and could be avoided by slowing the rate.

Eventually, of course, as the nephritic phase progressed to the point of renal failure, the albumin gradually ceased to be effective and three months before death the injections were, therefore, discontinued. At autopsy a search was made for unusual lesions that might be attributed to the prolonged administration of albumin and none could be demonstrated. The findings, as reported by the pathologists, were the usual ones associated with the terminal stage of a progressive nephrotic disease process of three years' duration.

There is no question that the daily administration of albumin, as a supplement to other therapeutic measures, maintained this child in a state of relative comfort and well-being to within a few months of death, thereby helping to prolong life by contributing directly to the preservation of a good nutritional state and by forestalling the complications that are associated with uncontrolled or uncontrollable edema.

While many other examples could be cited in which the beneficial effects of albumin infusions have been clearly demonstrated, this particular patient is mentioned because of the circumstances that made it possible to give the injections day after day without interruption for such a long period of time. In none of these other patients were harmful effects noted, as long as suitable attention was paid to the status of the heart and blood pressure, and to the patient's state of hydration, etc., and as long as care was taken with regard to the speed of injection.

CHAIRMAN GITLIN: There will not be time enough, if we do not move along. So, we will call on Dr. Heymann, who will discuss albumin and hyperlipemia. Then we will have general discussion and a coffee break.

C. Albumin and Hyperlipemia

DR. HEYMANN: I will not take long because I outlined last year some of the work which has been completed in the meantime and which I will report now.

These were studies which were stimulated chiefly by the elegant experimental studies of Rosenman, Friedman and Byers[5] who, in nephrotic rats in whom the disease was produced by anti-kidney sera, had prevented the flow of urine to the outside by ureteral ligation on one side, and by implantation of the ureter into the lower vena cava on the other side. They also studied the effect of intravenous infusions of albumin into nephrotic [illegible] venting effect [illegible] as indicating th[illegible] lipemia.

We have also used rats which were made nephrotic by anti-rat kidney serum obtained from rabbits.

In the first group of studies, using 19 nephrotic rats with well-established disease of one to 11 months duration, no correlation between severity of hypoalbuminemia and hyperlipemia was noted. If the hypoalbuminemia was the only cause for the nephrotic hyperlipemia, an inverse relationship between severity of plasma-albumin depletion and severity of the hyperlipemia could have been expected. That this has not been established does not eliminate, however, the hypoalbuminemia as one of several factors that may pathogenetically be involved in the elicitation of the nephrotic hyperlipemia.

In terms of time relationship, the development of hypoalbuminemia and hyperlipemia was studied in 26 rats, 2, 4, 6, 8 and 24 hours after the injection of anti-rat kidney serum, in 6 experiments. The increase in plasma-cholesterol concentration and fatty acid values preceded in all instances the decrease of serum albumin concentration below normal values. The serum lipids started to increase within 4 to 8 hours after the injection of anti-kidney serum, whereas the serum albumin values were found to be decreased usually not before the eighth hour after the serum injection. This, we believe, does not support the contention that hypoalbuminemia is the cause for the hyperlipemia.

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[5] Rosenman, R. H., Friedman, M. and Byers, S.O. The Causal Role of Plasma Albumin Deficiency in Experimental Nephrotic Hyperlipemia and Hypercholesterolemia. J. Clin. Invest., 35: 522, 1956.

In a last group of studies, 43 non-edematous nephrotic rats were used. Eight of these were bled every 6 to 8 days without being subjected to any injections. Another group of 8 nephrotic rats were bled at identical intervals and were given 4 cc. of saline solution by intraperitoneal injections twice daily for 10 days. A third group of 8 nephrotic rats received 4 cc. of protein-free urine twice daily which was obtained from healthy control rats. An additional group of 8 animals was injected with 4 cc. of protein-containing nephrotic urine twice daily, and a last group of 11 rats was again bled at identical intervals but injected intraperitoneally with 4 cc. of a dextran solution twice daily for 10 days. The intraperitoneal administration of protein-free rat urine decreased the hyperlipemia almost as regularly and markedly as when protein-containing nephrotic urines were used. One thus cannot be sure that urine obtained from control rats would not have decreased the hyperlipemia in Rosenman, Friedman and Byers' [5] experiments just as well as was noted after ureteral-vena cava anastomosis. The effect of dextran solutions on the experimental hyperlipemia was marked and regularly noted. It also was independent of hematocrit readings that were regularly obtained in three animals. The mechanism by which dextran decreases the nephrotic hyperlipemia is thus not clarified, but it seems that the hypolipemic effect of the infusion of albumin into nephrotic rats does not necessarily mean that the hypoalbuminemia has caused the increase in blood lipids. The nephrotic hyperlipemia is not very efficiently fixed. Frequent bleeding, even the administration of saline can cause some decrease in blood lipid concentration. We have shown also [6], for instance, that ethionine abolishes the nephrotic hyperlipemia completely within one to two days.

The coexistence of hypoalbuminemia and hyperlipemia is well-established in all forms of the nephrotic syndrome. We believe, however, that a causal relationship between them as yet has not been shown to exist. We also should not forget that hypoalbuminemia of comparable severity due to insufficient protein intake like in kwashiorkor, or as noted in hypocupremia and idiopathic hypoproteinemia, or as occasionally seen in chronic nephritis, are not necessarily associated with hyperlipemia.

Recently, we have just studied the development of hyperlipemia in the nephrotic renal disease induced by the aminonucleoside derived from Puromycin. Again, it develops prior to the development of hypoalbuminemia.

Within the last four months, Jerry Nash has in our laboratory developed a procedure to perform extensive plasmapheresis in rats, which is a difficult task in such small animals. Thus far, we only have three animals successfully treated in that fashion, three animals in which the albumin concentration has been reduced. The lowest value obtained in one of these was 2.2 gram per cent, without simultaneous occurrence of hyperlipemia.

CHAIRMAN GITLIN: Dr. Heymann's paper is open for discussion.

DR. EHRICH: I am not quite sure whether I understood the last experiment. It seems to me that the materials which were injected into the peritoneal cavity were

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[6] Heymann, W. and Hackel, D. B. Effect of Ethionine on Blood and Depot Lipids in Experimental Nephrotic Hyperlipemia. Proc. Soc. Exper. Biol. & Med., 92: 41, 1956.

inflammatory agents of various irritating capacity. The more irritating materials produced greater permeability of blood vessels, and hence more lipoproteins escaped from the plasma into the peritoneal cavity.

Wasn't it the inflammation which was produced in the peritoneal cavity, which caused the drop in the plasma lipoproteins?

DR. HEYMANN: I did not mention that all these agents were sterile. The solutions which we injected were well absorbed. They were gone within a day; and there were no gross changes in the peritoneal cavity which we observed at post-mortem examination. I do not believe that a loss of lipoproteins into the peritoneal cavity can have taken place to such an extent as to cause the marked reduction of plasma lipids noted.

DR. LANGE: You have not injected the urine intravenously?

DR. HEYMANN: No.

DR. RAPOPORT: What happens if you inject the fluid and promptly withdraw it? I am wondering about just the act of sticking them.

DR. HEYMANN: I do not know. You see, two out of the eight saline-injected animals had some definite reduction in total lipid concentration. I am always very much impressed that the concentration of lipids in plasma of nephrotic animals is anything but stable. You have to be very careful in the evaluation of results obtained. Even the removal of 2 or 3 cc. of blood within a three-to-four day period will result in a decrease of plasma lipid concentration.

DR. RAPOPORT: What you performed was, essentially, a Darrow-Yannet experiment. Nothing could be absorbed for some hours, until it was equilibrated. Dextrans would certainly cause distortion, and the concentrated urine even more so. I am just wondering whether the process of equilibration per se would lower the plasma lipids.

DR. HEYMANN: The mechanism of this effect is unclear. It only interested us in relation to the deductions pertaining to the hypoalbuminemia as a causative factor.

DR. MALCOLM HOLLIDAY (Pittsburgh, Pennsylvania): Did you ever use hypertonic salt solution?

DR. HEYMANN: No.

DR. METCOFF: This ought to increase the hyperlipemia, if anything; it ought not to decrease it, I should think. If you injected a hypertonic salt solution intraperitoneally and measured the lipid concentration in plasma shortly thereafter, it theoretically ought to increase because of withdrawal of water into the peritoneal cavity and the inability of the large lipid molecules to move promptly across the peritoneum.

DR. HOLLIDAY. Do you make your observations on change in blood lipids after three days? If so, absorption of peritoneal fluid has undoubtedly taken place. The temporary disequilibria due to injections intraperitoneally were observed two hours after injections.

DR. HEYMANN: Yes, the observations were three days after the onset of treatment.

CHAIRMAN GITLIN: Are there any other questions? If not, we can have a coffee break, I guess.

(The meeting recessed for twenty-five minutes.)

CHAIRMAN GITLIN: The next paper on the program is the question of interconversion of lipoproteins. Dr. Korn was supposed to give this particular paper; but, apparently, he has graduated into the senior class, because he says Dr. Rodbell did all the work. So Dr. Rodbell will give the paper.

D. The Interconversion of Lipoproteins

DR. MARTIN RODBELL (National Institutes of Health): I am going to talk this morning about the interconversion of lipoproteins. Dr. Gitlin has already mentioned a few aspects of the problem, and I hope I can amplify a little bit upon what he has talked about.

The question has been asked, in the past few years, as to the relationship each of the lipoprotein classes have to one another. That is, is there a conversion or any chemical relationship between each one of the density classes of lipoprotein which one finds in plasma?

It is known, through the work of Dr. Avigan[7] in our laboratory, and Dr. Bernard Shore[8] in the Donner Laboratory, that the high-density lipoproteins are not related to the density class having an Sf 0-10, both from a physical standpoint as well as from the fact that the proteins are entirely different in these two classes.

The question then arises as to the relationship the Sf 17-400 and chylomicron classes (the major classes responsible for the transport of triglyceride) have to the Sf 10-17 and the Sf 0-10 and the high density lipoproteins. We have heard some suggestive evidence this morning (which has been suggested by many people in the past) that there is a spectrum of lipoproteins ranging from chylomicrons to Sf 0-17, which is another way of saying that, as the triglyceride is removed from the plasma, one finds increasingly higher-density lipoproteins.

We know that the high density lipoprotein class is not converted to the Sf 0-10 or beta lipoprotein. However, it is not known whether chylomicrons and the Sf 20-400 are related to these classes. Some suggestive evidence that the Sf 20-400 class is related to the Sf 0-20 lipoproteins has been presented by Gofman and his collaborators[9].

[7] Avigan, J., Redfield, R., and Steinberg, D., Biochim. et Biophys. Acta, 20: 577, 1956.
[8] Shore, B., Arch. Biochem. and Biophys., 71: 1, 1957.
[9] Gofman, T. W., et al., Plasma, 2: 413, 1954.
Pierce, F. T., Jr., Metabolism, Vol. III. 142, 1954.

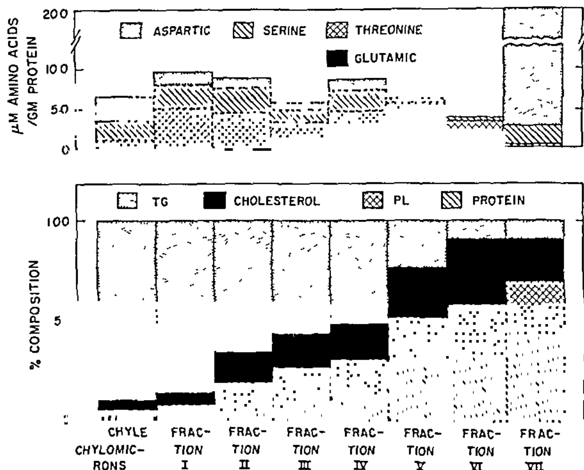


Fig 41 N-terminal amino acid and lipid composition of lipoproteins from human chyle and plasma. The isolation of the lipoprotein fractions was carried out according to the procedures described by Havel et al. *Fraction I represents plasma chylomicrons*, fraction II, Sf 400-400,000, fraction III, Sf 80-100, fraction IV, Sf 20-50, fraction V, Sf 10-17, fraction VI, Sf 3-8, and fraction VII, high density lipoproteins.

The underlying assumption, of course, which one makes in this work is that, if there is a spectrum of lipoproteins or if one class of lipoprotein is related to any other class, the protein moiety of the lipoprotein must be the same in the complete spectrum. If it is not, this would not allow for any continuum.

In the past few years, there have been developed in protein chemistry very elegant and very sensitive techniques for differentiating one protein from another based on one of the intrinsic properties of a protein. A protein, of course, is composed of amino acids which are linked through their carboxyl and amino groups to form a polypeptide chain. We have, therefore, at the end of the chain, a free carboxyl containing amino acid (C-terminal), and, at the opposite end, a free amino containing amino acid (N-terminal).

By tagging the N-terminal amino acid with a chemical marker, one can find out what amino acid is at the end of the polypeptide chain. In the experiments described today the free amino containing amino acids were coupled with dinitrofluorobenzene to give a colored dinitrophenyl derivative of the N-terminal amino acid.

Thus, if one finds that glutamic is the N-terminal amino acid (that is, the amino acid which has the free amino group) in one protein, and one finds aspartic as the N-terminal in another, these proteins, by definition, must be different.

Represented in the bottom half of Figure 41 is the per cent lipid composition of each of the types of lipoprotein-density classes which we have isolated from plasma and chyle.

Starting with chylomicrons from chyle, since this is the jumping-off point for triglyceride metabolism, one finds that they contain approximately 95 per cent triglyceride, some cholesterol, phospholipids, and a very small percentage of protein, about a half per cent in chyle chylomicrons.

The plasma chylomicrons (fraction I) are almost identical to the chylomicrons from chyle, at least in respect to the percentage of triglycerides and the percentage of cholesterol and phospholipids. The protein concentration apparently increases in the plasma chylomicrons.

Fraction II, sometimes referred to as lipo-microns or yellow chylomicrons, represents a particulate fraction which is smaller in size than chylomicrons and apparently has carotenoids associated with it. This material has a very close relationship to the chylomicrons. I will explain later why this is so true.

The lipid composition indicates that there is a smaller percentage of triglycerides in this component.

In the graph, fraction III represents Sf 40-100, fraction IV Sf 10-17, fraction VI Sf 3-8, and fraction VII the high density lipoproteins.

It is evident that most of the triglyceride-containing components in plasma are present from plasma chylomicrons down to about Sf 0-50 (fraction IV). When we proceed

into the Sf 10-18 and 3-8 class, we see that the cholesterol levels start to rise rather dramatically. In fraction IV the cholesterol and phospholipid ratio is less than one; as we get to fraction V, the ratio becomes greater than one. This follows for both the 3-8 and 10-17 classes. In the high density lipoprotein fraction we find that triglyceride is a minor component, cholesterol is somewhat of a minor component, and the phospholipid and protein seemed to predominate.

The top half of this graph records the yield of protein obtained, expressed in terms of micromoles of amino acid obtained per gram of protein, and the type of protein, represented by the N-terminal amino acid present, in each one of these lipoprotein fractions.

Much to our surprise, when we analyzed the protein moiety of the chylomicrons, we found three proteins present.

At first thought, we believed that perhaps they were just contaminants and this led us to go on, of course, and find out whether this pattern is repeated throughout the major triglyceride-containing lipoproteins.

After analyzing the plasma chylomicrons we found the same relationship. Again, we find that there are three proteins present and as in chyle chylomicrons, the three proteins contain threonine, serine and aspartic acid as N-terminal amino acids.

As you can see, there is a rather large shift in the relative ratio of the threonine protein in chyle chylomicrons to that of plasma chylomicrons, that is, the threonine protein seemed to predominate in the plasma chylomicrons whereas, in chyle chylomicrons, it is rather low.

I will not go into this to any extent but some very large changes in the physical properties of chylomicrons, once they get into plasma, have been observed, which may be related to this increase in the ratio of the threonine to aspartic and serine proteins[10].

We found the same pattern repeated in the lipomicrons. Again, we have the same relative relationship of one protein to another, in terms of concentration, and again three proteins. It was not until we analyzed the Sf 40-100 class that we began to pick up another protein, a fourth protein, containing glutamic acid as the N-terminal amino acid.

As we proceeded to analyze the Sf 20-50 class, we found a concomitant increase in the glutamic acid concentration in relation to the other three proteins. It was not until we analyzed the Sf 10-17 that we found a rather large increase in the glutamic acid containing proteins relative to the serine and threonine. The aspartic protein has disappeared completely.

The same phenomenon occurred in the Sf 3-8 class, where aspartic acid is gone and we find glutamic acid there in the greatest amount.

[10] Swahn, B., Scand. J. Clin. and Lab. Invest., 5: supplement 9, 1953.

The high density lipoproteins contained predominantly the aspartic acid proteins although we again found serine and traces of threonine.

This has been described before by Dr. Avigan[7] and Dr. Shore[8]. They also have found that the protein in the Sf 3-8 class contains primarily glutamic acid as the N-terminal amino acid.

They also find that there are traces of the threonine and serine components in each one of these fractions.

This rather complex picture, at first glance, seems rather confusing. However, if one looks at the graph, one finds that the proteins which are associated primarily with the triglyceride-containing lipoproteins are the serine, threonine, and aspartic-containing proteins. However, when we get to the cholesterol components, that is, the Sf 0-8 and 3-17 classes, the glutamic acid seems to enter the picture.

Of course, we all know that the cholesterol component in this area is quite elevated, and it would appear that there is some relationship between this glutamic-acid-containing protein and the cholesterol content.

Another aspect which seems to be quite clear is that there is a spectrum, if one wants to call it that, of proteins containing serine and threonine, ranging from chylomicrons down into the high-density lipoprotein class.

Right now, we do not know whether the aspartic protein represented in the chylomicrons is the same as the aspartic protein in the high density fraction (fraction VII). There is one disturbing aspect concerning this aspartic protein, and that is albumin. Human albumin also contains aspartic acid as its end-terminal amino acid, and it is possible that the Sf 20-400 and chylomicron aspartic protein is albumin and not the high density lipoprotein aspartic acid.

The serine, threonine and aspartic containing proteins are not connected together in any molecular entity. I have been able to separate, for instance, the threonine protein from the other two proteins, the aspartic and serine proteins. Therefore, it would appear that the three proteins are separate entities unto themselves.

This picture, of course, is complex; but it puts a new light, I think, on the metabolic patterns which people have observed in atherosclerosis and in nephrosis, where one gets alterations in the lipids of specific lipoproteins. As I said previously, the serine, threonine and glutamic proteins (or perhaps these three, because we do not know which one of these proteins is associated with triglyceride-containing components) may be necessary for the metabolism of the triglyceride.

I think we have reached another point in the study of the metabolism of lipoproteins in plasma, in which we can no longer discuss lipoproteins in terms of their Sf numbers or in terms of their density classes, but must think in terms of the types of proteins which are present in each fraction. I believe that if one were to make a concerted effort to study the changing patterns of these amino acid-containing proteins which I have just discussed, one may get a clearer picture of the complexities we quite often

see in such disease states as nephrosis, where one can get both cholesterol-changing patterns and triglyceride-changing patterns which may be a reflection of an alteration of metabolism of any one or all of the proteins found in the cholesterol and triglyceride fractions. Until we develop methods for the separation, and isolation and characterization of each one of these lipoproteins or proteins and determine what role they have in metabolism of the various plasma lipids, only then, I believe, will we know a little bit more about the metabolism of plasma lipoproteins. -- Thank you.

CHAIRMAN GITLIN: I would like to ask a question. You stated, Dr. Rodbell, that if a protein is converted one to the other, the end groups should be identical. I wonder whether this is absolutely necessary. Is it possible that, during the process of conversion, the process is not limited to the lipids, but that also there might be a stripping off of end groups, particularly the alpha-amino end groups?

DR. RODBELL: You are saying that, associated with the metabolism of the lipid moiety, you have a metabolism of the protein moiety, you may have a metabolism of the protein moiety. Of course, this we do not know; and I think it would be an interesting phenomenon, if true.

CHAIRMAN GITLIN: Has anyone actually studied the mechanism of action of the lipoprotein lipase on the protein moiety?

DR. RODBELL: No. The only thing we know is that, in the activation of coconut oil in the lipoprotein lipase activating system, the alpha lipoprotein (the high-density lipoproteins) are capable of activating, whereas we have had little success with the activation of triglycerides by the beta or the Sf 3-17 class [11].

CHAIRMAN GITLIN: This would suggest a specificity of the alpha lipoproteins; but it does not answer whether there is any stripping of any particular amino acid.

DR. RODBELL: No; it does not.

CHAIRMAN GITLIN: Have you tried stripping off that one terminal amino acid?

DR. RODBELL: To find out what the next two are? No, and actually until we can get some idea as to whether we can separate these proteins, which we are now approaching, I think we will defer that to later discussion and work.

This is a possibility, and one cannot deny that you could get some proteolytic activity, perhaps, in which you clip off, one by one, amino acids, and wind up with three proteins, all of which are identical, except that they have a difference in their end-terminal groups.

CHAIRMAN GITLIN: The other question I wonder about is, we have always taken it for granted that one specific end group implies one specific protein; that is, one end group implies one protein. When you actually think about this a little bit, I wonder how true it is, is there a possibility of a side chain?

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[11] Korn, E. D., J. Biol. Chem., 215: 15, 1955.

DR. RODBELL: Well, just speaking from historical conceptions, I would rule this out. However, there is always the possibility that you do have some side chain attached except for the fact that one finds a variable concentration, i.e., a relative concentration between the three, which suggests that these other amino acids are not related to one protein as a side chain and the fact that we can separate the threonine protein from the other two also suggests this.

CHAIRMAN GITLIN: Yes. Of course, the immunochemical analysis of these purified lipoprotein preparations has indicated that they are not purified lipoprotein preparations by any means. As a matter of fact, other proteins are included as well, even albumin.

DR. RODBELL: Yes. I know, in chylomicrons, it is apparently true that albumin can be found there.

I do not believe we can, at this point at least, make any definite statement as to the nature of these proteins until we can study them as separate proteins in themselves.

CHAIRMAN GITLIN: One good method I might suggest would be an immunochemical separation, because this can be done with specific precipitates, and knowing the end group of rabbit gamma globulin, by exclusion one can get the end group of the other proteins.

DR. RODBELL: The difficulty there is quantity. We need for analysis of these proteins, at least 10 to 20 mg. of protein.

CHAIRMAN GITLIN: We have done it with considerably less than that. I admit we have not done it on lipoproteins. However, this is very easily obtainable by immunochemical procedures.

DR. RODBELL: Yes.

CHAIRMAN GITLIN: Are there any other questions? I think this is a very exciting paper.

DR. BALLY: I would like to ask Dr. Rodbell if the composition of the chylomicra is the same from patient to patient?

DR. RODBELL: Not in the case of chylomicrons, but the lipomicrons represent a duplicate experiment, two different patients, both of whom contain this same pattern. But we had some difficulty in getting enough of this material. You see, the percentage protein in the chylomicrons, for instance, in plasma, is about 1 per cent; so you have to collect an awful lot of triglycerides or chylomicrons to do this.

But, we are attempting to repeat this on various individuals to see if we get the same pattern.

However, the suggestion is that we are going to find the same thing.

DR. JOSEPH KATZ (Los Angeles, California): I would like to reemphasize Dr. Gitlin's question. Do you believe that each terminal amino group represents a single protein? It very well could be a family of proteins with the same amino end group. Unless you determine also the terminal free carboxyl amino acids and find correspondence between the two groups you cannot decide this point.

DR. ROD[illegible] we can try to separate these things, [illegible] terminal end group as well and the [illegible] it has lipids on it. We do not even know whether all these proteins have lipids associated with them. One of them has to, but whether all three do, we do not know.

DR. KATZ: How do you separate various proteins?

DR. RODBELL: Of course, there are various methods. I would rather not go into the techniques we are utilizing right now. All I can say is we are rather optimistic at this point that we will be able to separate them. (Laughter)

That is being cagey, I guess.

CHAIRMAN GITLIN: I think you will succeed.

DR. RAPOPORT: On considering the whole spectrum of facts my feeling would be that the small amount of proteins present in the triglycerides represents an entirely different phenomenon than occurs in the protein-rich lipoproteins. My idea would be that the triglycerides actually are a vehicle for transport; and your chylomicrons have just gotten splashed with serum protein.

DR. RODBELL: Of course, this is a running argument. People say that chylomicrons are just globules of triglycerides floating around in plasma.

However, I think, again, the remarkable - I should not say remarkable, but the consistency in the pattern of the types of proteins which are associated with the chylomicrons and the other lipoprotein fractions, would suggest that the chylomicron is a lipoprotein, the same as the other fractions and it just has a larger amount of triglyceride associated with it, and takes the form of a particular structure rather than a soluble one.

Is that the question you were posing; or am I wrong? Were you doubting the existence of chylomicrons as lipoproteins?

DR. RAPOPORT: That is right.

DR. RODBELL: Of course, at this point one can only say that the data would suggest that the chylomicron is a lipoprotein, the same as all the other fractions; there is just more triglyceride.

DR. RAPOPORT: It would not seem to serve any useful purpose to have a particular tiny protein particle being transported by something. It can move on its own, of course; it is so small.

DR. RODBELL: Except that, capacity-wise, this would allow one to have perhaps more triglycerides while conserving one's proteins.

DR. H. W. SPATER (New York, New York): Can these chylomicrons be subjected to repeated washing to determine whether the protein components which may be soluble would decrease in amount during the washing procedure?

DR. RODBELL: The chylomicrons were washed at least eight times for analysis, until we obtained a constant protein concentration or percentage, I should say. Usually, after three washes, one obtains a constant protein percentage. After that, exhaustive washing does not seem to remove any more protein.

CHAIRMAN GITLIN: You could wash them indefinitely and still get many proteins *immunochemically. I believe you found at least four end groups. You can find at least* six or seven different proteins.

DR. RAPOPORT: What about the protein attached to the chylomicron in lymph?

DR. RODBELL: Well, it seems to show the same pattern as in plasma. It is true that we get a change in the distribution of the proteins; that is, we get an increase in plasma chylomicrons of the threonine component over that in chyle chylomicrons.

But there is a fair amount of evidence that very large changes occur in both the electrophoretic property of chylomicrons after they enter into plasma and their stability is greatly decreased after they get into the plasma. I do not know at this time what this is related to, however.

DR. HOLLIDAY: I take it the amino-acid patterns are quite consistent, from one batch of plasma to the other, within the same fraction.

DR. RODBELL: In the case of the lipomicrons, where we had two different patients, we get the same pattern; in the case of the Sf 40-100 class, we obtained this from the plasma of the individual from whom we collected the lipomicrons. You see, *they show the same kind of pattern (Fig. 41).*

We have not observed the same patient, however, over a long period of time; that is, taking a sample at one period, then waiting a month or so and taking it again to see whether it turns out to be the same. I think all these experiments will be very interesting to do in disease states, as in normal, to see whether there is any change occurring.

DR. HOLLIDAY: Does this represent a plasma pool?

DR. RODBELL: None of these represents a plasma pool.

I should say - I think this is an important point - that the chylomicrons we obtained from chyle came from a patient with a chylothorax. The chylomicrons which came from plasma were obtained from a patient with essential hyperlipemia.

This individual supposedly does not have any lipoprotein lipase or at least it is at very low levels.

The lipomicron fractions and the Sf 40-100, again, came from individuals who have essential hyperlipemia.

We obtained the Sf 20-50 fraction from a supposedly normal individual at the NIH blood bank. The Sf 10-17, 0-10, and high density lipoproteins came from supposedly normal individuals from the blood bank, taken from random samples. In the case of the Sf 3-8's, we pooled samples. However, we have not taken samples of large populations to see whether we would get any variation. I suspect we will get variation in terms of concentration of any one of these components, that is, we may find another individual who instead of having 80 per cent of the proteins containing glutamic in the Sf 3-8 may have only 50 per cent having glutamic, and perhaps more of the serine.

But this may be a reflection of the triglyceride metabolism changes within the individual.

DR. BALLY: How many times do you have to spin the 1.21 density fraction in order to get rid of all of the albumin?

DR. RODBELL: We centrifuge, conventionally, at 1.21 density in the 40 rotor; then we resuspend this material and centrifuge it another 24 hours in fresh solvents in the 40.3 rotor, and take a very high cut.

This is all the washing we do for that particular cut.

DR. BALLY: But you have not checked that fraction for albumin thereafter, have you?

DR. RODBELL: No, we have not.

However, Shore has demonstrated quite conclusively that albumin and the high density lipoproteins are not the same proteins.

CHAIRMAN GITLIN. I think it would be best to go on to the next paper, which is to be presented by Dr. Robert Gordon.

E. Unesterified Fatty-Acid Metabolism

DR. ROBERT GORDON (National Institutes of Health): I presume I do not need to apologize to this conference for not talking about nephrosis, since Dr. Rodbell was permitted to discuss biochemistry only. What I am going to discuss primarily is the physiology of unesterified fatty acids in normal subjects. The experiments involved have been done by a number of people besides myself: Miss Cherkes, Dr. Donald Fredrickson (who is lurking in the corner over there), his assistant, Mr. Ono, and Dr. DeWitt Goodman.

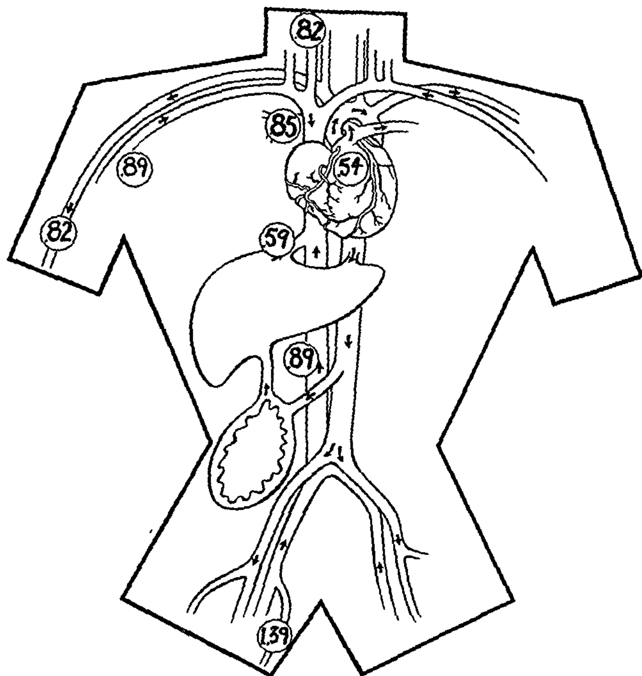


Fig. 42: Site and concentration of unesterified fatty acid analyses in arteriol and venous blood obtained by cardiac catherterization.

The first slide, Figure 42, will show what we have found in subjects who are not quite normal. These are patients who come into the Heart Institute's surgical service for cardiac catheterization. They may have congenital or acquired cardiac defects, but I think we can regard them as normals from the point of view of lipid metabolism. In the course of performing catheterizations, it is not unusual to obtain samples from a hepatic vein, coronary sinus, or other specific vein and, by analyzing these blood samples, along with an arterial blood sample taken at the same moment, for their content of unesterified fatty acid we have been able to measure arterio-venous differences of unesterified fatty acid. The analytical method we have used in obtaining these data has a standard error of about .02; so you will see that the A-V differences are on the order of ten times the standard error, thus the individual measurement is usually significant. These values have been obtained repeatedly; and I think there can be no doubt that the differences are more than just random analytical error.

The value appended to the brachial artery is the mean of unesterified fatty acid content in the whole group of these patients. These people are all terrified of the procedure; and they are all fasting, both of which influences tend to make this value fairly high compared with some other normal figures you may have seen. Blood obtained from the hepatic vein will, in general, have less unesterified fatty acid in it than does the arterial sample. The figure of 0.59 is not actually the average of all hepatic vein samples which we studied. The number is so chosen that the difference between it and the arterial mean, 0.33, is equal to the average A-V difference encountered in the eight or so subjects in whom we measured an arterial-hepatic vein difference.

Coronary sinus, which is represented here, also has a much lower content of unesterified fatty acid than does the artery. Peripheral veins tend to have somewhat higher values, the greater saphenous vein having the highest of all. The internal jugular vein bringing blood back from the brain, and the pulmonary artery, are not significantly different from peripheral arterial values.

Putting this all together on the figure is artificial, because we never measured these values all at once on one patient. We do, however, develop a picture of unesterified fatty acids entering the circulation in the periphery, best exemplified in this greater saphenous vein which, as you know, is a superficial vein bringing no blood back from skeletal muscle, but only blood from adipose tissue and the skin, and leaving the circulation in myocardium, in liver and, in all probability, in other tissues, though myocardium and liver are really the only two from which we have obtained good repeated samples in human subjects.

Table 11 is one of three which we can go through in fairly rapid succession. These are actual UFA values as measured on one patient, duplicate determinations on the artery and vein (in this case, the coronary sinus) and the A-V difference. Given the figures for oxygen content on the same blood samples, we can make a rapid comparison, based on simple stoichiometric principles, with the amount of oxygen that would be required for the complete oxidation of the observed A-V difference of fatty acid [12, 13]. Each of these oxygen contents is expressed per 100 ml. of blood flow.

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[12] Gordon, R. S., Jr., and Cherkes, A., Unesterified Fatty Acid in Human Blood Plasma. J. Clin. Invest. 35: 206, 1956.

[13] Gordon, R. S., Jr., UFA in Human Blood Plasma. II. The Transport Function of UFA. J. Clin. Invest. 36: 810, 1957.

TABLE 11

A-V UFA DIFFERENCES

(Coronary Sinus)

Patient	Arterial	Venous	Difference	Per Cent O_2
S.M.	.94 .98	.76 .75	.20	42

Repeat 30 minutes after glucose and insulin intravenously

	.32 .30	.32 .29	*none*	

The oxygen actually extracted by the heart was about 2-1/2 times as great as the amount which would be required for the complete oxidation of the fatty acid simultaneously extracted so that 42 per cent of the oxygen, we can assume, was being utilized by this myocardial tissue for the oxidation of this fatty acid.

With the catheter remaining in place, this subject was given glucose and insulin intravenously. The reason for the insulin was merely to decrease the time we would have to wait before getting our second samples. After this treatment, when the subject is oxidizing carbohydrates primarily, the A-V difference is gone. We have not calculated the oxygen extraction ratio for obvious reasons. Both arterial and venous UFA levels are lower than they were; but the important thing is that the extraction has ceased.

Table 12 will show the same experiment done for the hepatic vein. Here we have obtained samples from three patients, as it is easier to persuade the responsible physicians to allow us to maintain a catheter in place for 30 minutes when it is in the hepatic vein. Here we have differences, in each case abolished by the administration of glucose and insulin intravenously.

TABLE 12

ABOLITION OF A-V DIFFERENCES BY GLUCOSE AND INSULIN I.V.

Patient	Vein	G & E	Arterial	Venous	Difference
J.B.	H.V.	pre	.68	.63	.05
		post	.10	.10	.00
W.H.	H.V.	pre	.89	.78	.11
		post	.25	.25	.00
A.J.	H.V.	pre	.42	.31	.11
		post	.08	.08	.00

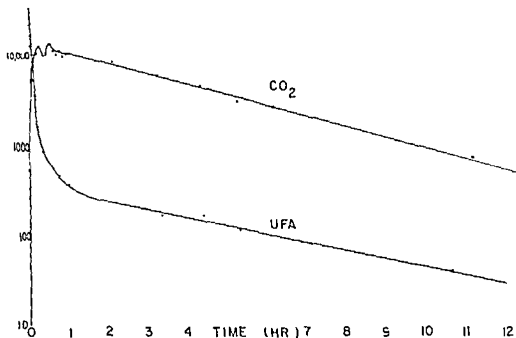


Fig 43 Specific activity of labelled unesterified fatty acid following intravenous injection of C^{14} labelled palmitic acid.

CELLS

PLASMA

U.F.A.

E.C.F

HCO_3^-

EXPIRED AIR

CO_2

TABLE 13

ABOLITION OF A-V DIFFERENCES BY GLUCOSE AND INSULIN I.V.

Patient	Vein	G & E	Arterial	Venous	Difference
G.E.	S.V.	pre	.46	1.09	-.63
		post	.16	.19	-.03
R.S.	S.V.	pre	.39	.82	-.43
		post	.22	.20	.02
I.S.	S.V.	pre	.36	.50	-.14
		post	.13	.12	.01

Table 13 will show that the negative A-V difference in the saphenous vein is likewise abolished; all these post-glucose A-V differences are insignificantly different from zero. Thus, the administration of carbohydrate has put an end to the transport of fatty acid from the periphery to the viscera as unesterified fatty acid.

Another line of investigation which leads to more or less the same conclusion, namely that unesterified fatty acid is transported and oxidized, is made possible by the availability of radioactive tracers [14]. In Figure 43 are presented some kinetic data. We have taken palmitic acid labelled with carbon-14 in the carboxyl group, dissolved this in human serum albumin as a sodium salt; so that we have a labelled unesterified fatty acid which corresponds chemically, as far as we know, with that which is circulating in vivo. At time 0, this is injected into the vein of a fasting subject, who has been deprived of food at least overnight and, in many cases, almost 24 hours. Then we do serial determinations of the specific activity of the unesterified fatty acid in circulation and of the expired carbon dioxide.

The unesterified fatty acid disappears from the plasma, distributing itself in some larger compartments with extraordinary rapidity. The half-time of this slope, which in Figure 43 can hardly be differentiated from the vertical axis, is about two minutes in most normal subjects. At the same time, the label appears with equal rapidity in the expired carbon dioxide. This means that the tracer substance has gone out of the plasma into some compartment from which carbon dioxide is being derived. After about an hour, this distribution process is largely complete; and the plasma specific radioactivity has dropped by a factor of very close to 100. From this point onward, the plasma unesterified fatty-acid slope and the expired carbon dioxide slope are almost parallel. This slope, we feel, represents the metabolism of the fatty acids in the extravascular functional pool into which they distribute themselves within the first hour.

Figure 44 shows our present rough conceptual scheme of the nature and size of these functional compartments. First, there is a very small functional compartment

[14] Fredrickson, D. S. and Gordon, R. S., Jr., UFA in Human Blood Plasma. III. Distribution and Metabolism of C^{14} labelled UFA in Normal Subjects. (In prep.)

of circulating unesterified fatty acids connected through a rather broad channel, which is reversible, with an extravascular and almost certainly intracellular functional compartment of lipids. I do not specify this compartment as unesterified: The fatty acids may have become esterified in the course of crossing this boundary; but, if so they can become unesterified and return to the plasma. In the intracellular compartment, oxidation is occurring with production, of course, first, of bicarbonate, and then of expired CO_2. This compartment is being maintained by an influx of unesterified fatty acid from peripheral adipose tissue which enters by way of the circulating plasma. Our data strongly suggest that no significant fraction of circulating UFA can re-enter the depot fat from which it came. In terms of their gross size, these compartments contain roughly 0.5 gram of fatty acid in the plasma, and roughly 50 grams in the tissue pool. Of course, the depot fat is measured in kilograms, depending upon the size and shape of the subject.

Table 14 will show some derived values calculated from the analysis of kinetic curves such as I just showed you in two normal fasting subjects. The half-time for the second component of the disappearance of plasma unesterified fatty acid was 110 minutes in subject U.W., 260 in M.B. The half-time for the corresponding carbon dioxide disappearance was 139 and 175. These are not different enough to be of great significance, since this type of measurement is subject to considerable error. The pool into which the plasma unesterified fatty acid was distributed (the big pool shown in Figure 44) contained in this subject 250 milliequivalents of fatty acid - this would be about 50 g. - and, in the other subject, 232. The turnover, the number of milliequivalents oxidized from the tissue pool per day, gives two figures which are somewhat discrepant, but still not by any means an order of magnitude apart.

TABLE 14

CONVERSION OF C^{14} PALMITATE TO $C^{14}O_2$ IN FASTING NORMAL HUMANS

	V.W.	M.B.
UFA $T_{1/2}$ (min)	110	260
POOL (mEq.)	250	232
TURNOVER (mEq/d.)	2260	890
CO_2 $T_{1/2}$ (min)	139	175
POOL (mEq.)	4350	6050
(" ÷ 16)	272	378
TURNOVER (mEq. C_{16}/d.)	1960	2160

The same estimation of turnover can be made by analyzing the CO_2 curves. In subject V.W., we have very good agreement; these two figures should be identical if our experimental techniques were perfect, and our model absolutely applicable. With M.B. there is somewhat larger disagreement. This sort of disagreement would be expected to arise if this subject had been making some carbon dioxide from a source other than our intracellular pool. In other words, if he were oxidizing some carbohydrate and making unlabelled carbon dioxide, we would get just this discrepancy.

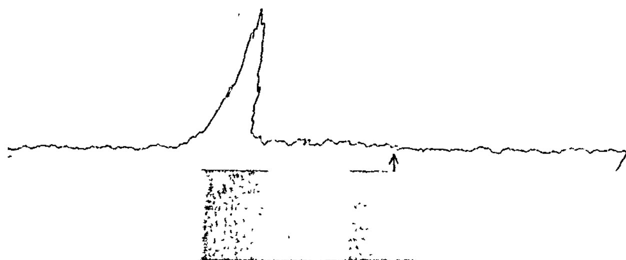


Fig. 45· Paper electrophoresis of normal rat serum equilibrated in vitro with labelled sodium palmitate. Virtually all the radioactivity is associated with the albumin fraction shown under the peak at the left of the stained paper strip.

These results can be taken as evidence that the plasma unesterified fatty acid, plus the pool of lipids with which it is in equilibrium, are the predominant precursors for expired carbon dioxide in fasting subjects. One can calculate the calorie eqivalence of the oxidation of this much fatty acid; we come out with a figure which, in general, has been in the range of 3000 to 4000 calories per day derived from the oxidation of unesterified fatty acid. Slightly over two calories are evolved per milliequivalent of fatty acid of average molecular weight which is oxidized.

What does this have to do with nephrosis? So far, we have done one study of this sort in a nephrotic subject. He presented the typical clinical picture of the disease, with a plasma albumin of about 0.5 g per cent and creamy serum. He was a young lad with no evidence of renal functional impairment. I have not prepared a graph of those data, because it would have looked very like the kinetic graph I showed you first. The disappearance of unesterified fatty acid from the plasma into the extravascular compartment had a half-time of two minutes. The later, slower slope of the CO_2 and unesterified fatty acid was approximately 180 minutes. However, the ratio of the specific activity of expired CO_2 to the specific activity of plasma UFA at Time 0 was greater than normal, indicating less dilution of the tracer with endogenous UFA, and perhaps a quantitative impairment of endogenous UFA catabolism.

This brings up the last subject; namely, the physical chemistry of the interaction of unesterified fatty acids with albumin. Figure 45 represents an experiment performed in a rat, but I think the situation is very much the same in humans. If we take normal rat serum and add to it, in vitro, a small amount of sodium palmitate, again labelled with C^{14}, and allow the palmitate ion to interact with the serum albumin, then run a paper electrophoresis, we separate gamma globulin, alpha globulin, beta globulin; and albumin. The rat does not have two alpha globulin sub-fractions like the human. If we scan for radioactivity we [illegible] paper. There is a trace [illegible] represents absorption of a [illegible] When we come to the albumin band, we find virtually all the radioactivity present in this component. Since unesterified fatty acid normally travels through the plasma as a passenger aboard albumin, we had reason to believe that the up to tenfold decrease of albumin concentration which is found in nephrosis and some other disturbances might play some role in disturbing lipid metabolism. The tracer experiments in the nephrotic subject were carried out in the hope of finding supporting evidence for this idea.

DeWitt Goodman has been studying the interaction of unesterified fatty acids with albumin in a purified, simplified system in vitro. The next two tables will explain the experimental methods and the conclusions which can be drawn from investigations of this type.

Two fatty acid solutions, one with and one without albumin, in equilibrium with the same concentration of fatty acid in heptane, are in equilibrium with each other. This is a basic thermodynamic concept. In this experiment, one can conclude that the activity or chemical potential, of fatty acids in the solution without albumin equals the chemical potential of fatty acids in the solution with albumin, and, if the solution with albumin is

in internal equilibrium, the chemical potential of fatty acid which is in the free state, and that which is bound in albumin, *must be the same*.

TABLE 15

PRINCIPLES OF PARTITION ANALYSIS

Principle: Two fatty acid solutions, one with and one without albumin, in equilibrium with the same concentration of fatty acid in heptane are in equilibrium with each other.

Conclusion: For two such solutions, the activity of the fatty acid in the solution without albumin equals the free (unbound) fatty acid activity in the solution with albumin.

Here, then, is a technique which can be applied like the dialysis-equilibrium technique to the study of the binding of small molecules to protein. In dialysis equilibria, one examines two solutions separated by a dialysis membrane through which the small molecule can pass and the albumin cannot. In the case of the higher fatty acids, no such membrane has been found, but the heptane solution serves as a bridge, as it were; it takes the place of the dialysis membrane, and assures us that two solutions, one with albumin and one without, are in thermodynamic equilibrium with each other as regards the fatty acid component.

TABLE 16

TECHNIQUE OF PARTITION ANALYSIS

Method: Equilibrate the fatty acid between two phases, with and without the presence of albumin.

Phases and conditions:

Upper Phase: n-heptane
Lower Phase: Phosphate Buffer
Iónic Strength 0.16
pH 7.45
Temperature 23°C.

Table 16 outlines the experimental method. The upper phase is pure normal heptane; the lower aqueous phase is phosphate buffer with pH and ionic strengths which are essentially physiological. The temperature is 23°C and, to this extent, we have a condition slightly different from the in vivo situation. It is possible to describe the binding of fatty acids to human serum albumin in terms of equilibria in which one reactant is the free unesterified fatty acid and the other reactant is a site on the surface of albumin. Each albumin molecule contains two sites of one type, five sites of a second type and, roughly, twenty sites of a third type. One of the assumptions is that these sites are all different and independent; in other words, if a fatty acid combines with one site there is no change in the affinity of another site for the next fatty acid

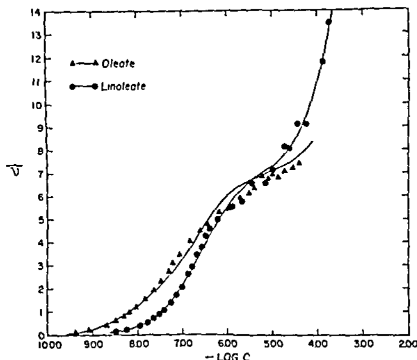


Fig. 46 Titration curve of fatty acids. The ordinate represents the number of molecules of fatty acid bound per molecule of albumin. The abscissa is the negative logarithm of the concentration of free or unbound fatty acid. The titration curves of oleate and linoleate are shown.

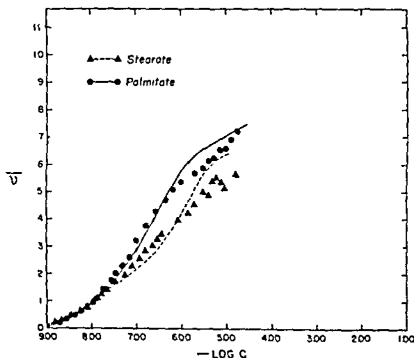


Fig. 47 Titration curve for stearate and palmitate. Symbols and units are similar

molecule. Table 17 presents a summary of results without going through the mathematical analysis, which is rather complex. The data are association constants which fit a mass law expression of the reaction of free, unesterified fatty acids with sites on the albumin molecule to yield a complex. The association constants for the first class of sites are very high, compared with the binding of ions of other types to albumin, which has been studied extensively in the past with association constants ranging from 10^2 up to 10^4. The sites of the second class, which are more numerous, have considerably less affinity for the fatty acids; and the third class of sites are essentially insignificant in terms of their binding capacity.

TABLE 17

FATTY ACID - ALBUMIN INTERACTION

Association Constants for three classes of binding sites

$n_1 = 2$; $n_2 = 5$; $n_3 = 20$

	k_1	k_2	k_3
Laurate	$1.6x10^6$	$2.4x10^5$	$6x10^2$
Myristate	$4.0x10^6$	$1.4x10^6$	$2x10^3$
Palmitate	$6.0x10^7$	$3.0x10^6$	$1x10^3$
Stearate	$8.0x10^7$	$8.0x10^5$	$1x10^3$
Linoleate	$1.3x10^7$	$2.5x10^6$	$2.5x10^3$

The curve in Figure 46 is a titration curve. You may not recognize it as such immediately; but, if you equate this experiment with the titration of a protein with acid or base, the symbol $\bar{\nu}$ is the number of molecules of fatty acid bound per molecule of albumin. It has the same significance as the symbol B which is used in titration curves; B is the number of molecules of acid or base bound. Along the abscissa is plotted the negative logarithm of the concentration of free or unbound fatty acid, derived from the concentration in heptane which is in equilibrium. It is equivalent to pH in the titration of proteins with acids. The result is a titration curve which is not dissimilar to the titration of a protein with acid or base.

At a $\bar{\nu}$ value of one (a ratio of one fatty acid molecule per albumin molecule in the aqueous phase) the system is in equilibrium with a free fatty acid solution between 10^{-7} and 10^{-8} molar. This illustrates the reason why I do not like the term "free fatty acid" when dealing with plasma UFA concentrations. Most of the circulating UFA is albumin-bound. Figure 47 shows very similar titration curves for stearate and palmitate. These, with oleate, are certainly the fatty acids of most importance in normal fat transport[15].

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[15] Goodman, DeW. S., Interaction of Human Serum Albumin with Long-Chain Fatty Acid Anions (in prep.).

The last slide in this group is the one which Dr. Baxter showed earlier (Fig. 40). Three nephrotic patients with very little serum albumin are shown. Albumin values are expressed in terms of millimols per liter, which works out better for the estimation of $\bar{V}$. In the particular nephrotic shown at the top, there is a molar ratio of approximately 6:1 during the period before treatment was given. In the second nephrotic, it is on the order of 3:1; and, in the third case, the molar ratio is close to the normal value of 1:1. Returning to the titration curves, let us see what is implicit in a molar ratio of 6:1.

The concentration of free, unbound fatty acid is on the order of 10^{-5}, whereas in the normal subject, it is on the order of 10^{-8} (Table 18). Here is one variable, the concentration of free, unbound fatty acid, which may be 1000 times higher in the nephrotic than in the normal. Please take this calculation with a grain of salt. There are in the human subject lipoproteins, red cells, and all manner of other things which might buffer this curve, but it still may be possible that a change in fatty acid to albumin ratio which does not look impressive (perhaps two-, three-, four-, or five-fold) may produce much greater changes in a dependent variable whose physiological significance we do not yet know, but which may be considerable.

If, then, we can correct the situation by administration of albumin, and thus change the molar ratio of UFA to albumin, we produce a change in the chemical potential of the unesterified fatty acid, which may, in turn, affect some synthetic mechanism. Clearing of lipemia could be a consequence. I think this speculation would be concordant with Dr. Gitlin's hypotheses. The albumin does not participate merely as a reactant in degrading the lipoproteins, but, by holding the unesterified fatty acids at low chemical potential, may control mechanisms for the synthesis and degradation of lipoproteins which may be closely related to the hyperlipemia. Thank you.

TABLE 18

ESTIMATED FREE UFA CONCENTRATIONS

NORMAL:	(Alb.)	=	6×10^{-4}		
	$(UFA)_{total}$	=	6×10^{-4}		
	$(UFA)_{free}$	=	2×10^{-8}		
NEPHROTIC	(Alb.)	=	6×10^{-5}		
	$(UFA)_{total}$	=	3.2×10^{-4}		
	$(UFA)_{free}$	=	4×10^{-6}		
ASSUMPTIONS	k_1	=	5×10^{7}	n_1	= 2
	k_2	=	5×10^{5}	n_2	= 5

No sites for the binding of UFA except these two classes on albumin need be considered.

CHAIRMAN GITLIN: Thank you very much, Dr. Gordon.

I would like to ask one question. That is, have you demonstrated that the degradation of the nonesterified fatty acids is a first order reaction? Not that a given amount will disappear per unit time but, rather, if there are two different concentrations of unesterified fatty acids, one very high and one very low, would the fractional rate of degradation be the same?

DR. GORDON: On the contrary we have demonstrated that it is not the same. Low concentrations of circulating UFA occur in subjects fed carbohydrate; they convert less UFA to CO_2 per unit time.

CHAIRMAN GITLIN: I think this would bear very much upon what I have said, because of the high concentration of fatty acid in the nephrotic individuals.

DR. GORDON: We cannot apply our studies on normal subjects to nephrotics. The total concentration of unesterified fatty acid, which is the only measurable variable, in a fasting subject will be on the order of .6 to 1.0 milliequivalents per liter when we do one of these turnover studies. We have done a moderate number of these investigations in normal subjects who have been given carbohydrates in such a way as to reduce the level to something on the order of .2 to .4. By preparing a scatter plot of the rate of disappearance from the plasma, the first rapid slope in that disappearance curve, against UFA concentration, we get the impression that there is a reciprocal relation between the half time and the fatty-acid concentration. It comes out in such a way that the total turnover, the quantity in grams of unesterified fatty acids leaving the plasma compartment per minute, is close to constant. We are not sure that it is exactly constant. The limiting factor may be the capacity of endothelium or cellular membranes to transmit this material, so that the process of leaving the plasma definitely is not first order. After the UFA gets out into the larger cellular compartment, the rate of oxidation is very clearly a function of the extent to which the organism is relying on fat as a metabolic substrate. That rate is reduced almost to zero in rats by carbohydrate feeding, and is brought to a maximum by fasting.

CHAIRMAN GITLIN: In the same fasting individual, can you increase his unesterified fatty acid by injecting a solution of fatty acids?

DR. GORDON: No, that experiment cannot be performed safely. We cannot conceivably administer fatty acid at a rate which is even 1 per cent of the rate at which the subject administers it to himself, so that we cannot manipulate the UFA concentration. The reason is that unesterified fatty acid unbound to albumin is intensely toxic and hemolytic; and that, at the point of any needle we must use, we would produce fatal reactions without altering the average circulating concentration.

DR. BALLY: Are you not giving appreciable quantities of UFA's when you infuse albumin?

DR. GORDON: You do administer some unesterified fatty acid along with albumin; but, if you consider this in terms of how many millimols you actually give, it is equal to about one or two minutes normal turnover. So, within a minute after giving the

albumin, the unesterified fatty acids which came in with it are gone; and, from there on, for the rest of the 24-hour period, this exogenous fatty acid could not, to my mind, have any measurable effect.

DR. JOSEPH KATZ (Los Angeles, California): You made the statement that all CO_2 of these two fasting subjects was derived from fatty acids. It seems from your table that there is considerable difference between the two subjects. I also wonder what the contribution to CO_2 by glucose, amino acids, etc., would be.

DR. GORDON: By "all," I mean plus or minus 25 per cent.

If you plot those data - for example, $C^{14}O_2$ output after the administration of radioglucose or fructose, on the same scale that we have used for our CO_2 curves, they would look very different. The rate at which the isotope appears in expired CO_2 is four- or five-fold slower when you administer radioglucose than when you administer unesterified fatty acid. But the rate of appearance of $C^{14}O_2$ is not the only significant factor. The size of the pool is very important also, and I think the data we have and those of others obtained after administration of radioactive carbohydrates to human subjects are entirely compatible.

DR. KATZ: What type of curve would you get if, instead of free fatty acid, you used the esterified labelled compound?

DR. GORDON: We have not done experiments of this sort in humans. If you say a labelled esterified fatty acid, you have not described an experiment adequately until you can specify exactly in what form this labelled ester is to be given. Dr. Fredrickson, who is here, might want to comment very briefly on administration of chylomicrons which contain a labelled esterified fatty acid. This experiment has been done repeatedly in dogs, not in humans, because in the case of chylomicrons, we cannot prepare a substrate which we are entirely convinced is safe for administration.

DR. RAPOPORT: From your hypothesis, would you be willing to speculate on how a patient with hyperlipemia and normal albumin might fit in?

DR. GORDON: Our experiments so far have not revealed any significant abnormality in these patients. The mole ratio of unesterified fatty acid to albumin is essentially normal or differs only slightly in patients with essential hyperlipemia, so that this line of reasoning does not yet lead us to an explanation f the basic metabolic defect in essential hyperlipemia. But we cannot clear the lipemia in these cases by administering albumin; whereas, as Dr. Baxter showed, the administration of albumin, enough to correct the defect in albumin in nephrotics, does seem to correct their defect in lipid metabolism.

DR. RAPOPORT: Where does heparin fit in? Would you be willing to speculate on that?

DR. GORDON: I do not think heparin is intimately related to this lipemia. I am afraid that another conference, which could easily occupy several days, would be required to do justice to that question. I think we had better let it go for the moment.

DR. DONALD FREDRICKSON: (National Institutes of Health): We have compared the metabolism of palmitic acid-1-C^{14} injected intravenously as UFA bound to albumin and as triglyceride fatty acid contained in chylomicrons. The results with UFA are essentially the same as those we have obtained in humans. Labelled chylomicrons have then been injected into dogs in comparable nutritional states. The triglyceride is rapidly removed from the plasma. We have obtained additional evidence that intravascular hydrolysis is not essential for this "clearing". During this process the UFA in the plasma becomes radioactive rapidly. The maximum specific activity is reached after about two-thirds of the chylomicron triglycerides have left the blood. The UFA radioactivity then rapidly decreases. Meanwhile the radioactive carboxyl carbon is appearing in the expired CO_2. When the specific activities of the plasma UFA and expired CO_2 obtained after the injection of C^{14}-palmitate as UFA and as chylomicron triglyceride fatty acid are compared, two relationships stand out. One is the similar rate of excretion and specific activity of the $C^{14}O_2$ after either substrate is injected. The other is the much lower specific activity in blood UFA after chylomicron injection. The conclusion appears justifiable that retransport of the chylomicron triglyceride fatty acids as UFA in the plasma is not essential to their oxidation. Thus triglyceride may go directly into those intracellular "areas" with which the plasma UFA is also in equilibrium. In the fasting animal, a large part of the chylomicron triglyceride fatty acids are not stored, but are rapidly oxidized.

CHAIRMAN GITLIN: Are there any other questions?

If not, Dr. Bally will speak on the influence of the pituitary on hyperlipemia of nephrosis in rats. Dr. Bally!

F. Influence of the Pituitary and the Adrenal on Hyperlipemia of Nephrotic Rats

DR. PETER BALLY (Peter Bent Brigham Hospital, Boston): I am afraid that what I will present is a rather naive anticlimax to Dr. Gordon's beautiful paper.

I am going to discuss briefly three studies which were done respectively in Dr. E. Kendall Emerson's Laboratory at the Peter Bent Brigham Hospital, in Dr. John Merrill's Laboratory also at the Peter Bent Brigham Hospital and the third study together with Dr. Shosaku Numa in Dr. Oncley's department at Harvard Medical School.

The first study* is an attempt to evaluate the synthesis of phospholipids in the so-called "liver-plasma pool" in rats with nephrotoxic serum (NTS) induced nephrotic syndrome (NS). All rats developed severe proteinuria within a few hours after NTS injection.

Table 19 shows the in vitro incorporation of inorganic phosphate P32 into the phospholipid phosphorus of liver slices obtained from nephrotic rats on the third and

*This study was made possible through a U.S. Public Health Service Grant (No. A-137) and a grant from the American-Swiss Foundation.

fifth day after NTS injection and from control animals injected with complement-free normal rabbit serum. The liver slices were incubated in a Krebs-Ringer bicarbonate buffer at pH 7.4 and 37°C. for 3 hours. The incorporation of P32 has been expressed as relative specific activity (SA), i.e., phospholipid phosphorus SA as related to inorganic phosphorus SA.

TABLE 19

INCORPORATION OF PHOSPHATE P^{32} INTO LIVER PHOSPHOLIPID-PHOSPHORUS IN VITRO

(Expressed as relative specific activity: Phospholipid-Phosphorus Specific Activity Divided by Inorganic Phosphorus Specific Activity)

	No. of animals	Days after inj. of NTS	Relative specific activity
Nephrotic	8	3rd	0.0236 $\pm$ .0072
Control	10	---	0.0320 $\pm$ .011
Nephrotic	5	1 day	0.0211 $\pm$ .0072
Control	4	---	0.0250 $\pm$ .0051

(No significant difference for $p = 0.1$)

If inorganic phosphate phosphorus is assumed to be a sufficiently immediate precursor of liver phospholipids to permit conclusions with regard to overall phospholipid synthesis in the liver-plasma pool, these data would indicate the absence of a significant change of phospholipid synthesis in the nephrotic liver, which presumably, at least under normal conditions, is the sole source of plasma phospholipids[16].

Similar studies in vivo, in which P32 labelled inorganic phosphate was injected intraperitoneally at various stages of the nephrotic state also have failed to show a highly significant difference in the incorporation of P32 into liver phospholipid phosphorus 2½ and 4 hours after injection of the labelled phosphate although there seemed to be a tendency for the nephrotic rats sacrificed after 2½ hours to incorporate P32 into plasma phospholipids at a somewhat lesser rate than their controls in the early stage of the disease. This statistically significant difference (p less than .1) was not apparent if the animals were sacrificed 4 hours after injection of the labelled phosphate. However, it must be remembered that these animals cannot be considered in a steady state with respect to their lipid metabolism while they are developing hyperlipemia and hence do not fulfill Zilversmit's criteria for an in vivo turnover determination[17].

[16] Fishler, M. C., Entenman, C., Montgomery, M. C. and Chaikoff, I. L., The formation of phospholipids by the hepatectomized dog as measured by radioactive P32. I. The site of formation of plasma phospholipids. J. Biol. Chem. 150: 47, 1943.

[17] Zilversmit, D. B., Entenman, C. and Fishler, M.C., On the calculation of turnover time and turnover rate from experiments involving the use of labelling agents. J. Gen. Physiol. 26: 325, 1943.

Similar experiments were carried out with regard to the incorporation of C14 from acetate-1-C14 into the digitonin precipitable fraction of liver lipids in vitro. Rats were sacrificed at intervals after NTS administration, the livers were excised, sliced and incubated under the conditions described before. Results obtained varied within a wide range. However, no significant difference in the incorporation of acetate carbon into liver cholesterol was found between nephrotic rats and their controls at an early stage of the disease (prior to four days after NTS injection), whereas incorporation seemed to be moderately depressed subsequently. These results were in agreement with data reported by Drabkin[18], although they were not as clear-cut.*

TABLE 20

THE PHOSPHOLIPID CONTENT OF THE LIVER PLASMA POOL

	mg.
Control:	187 ± 32
Nephrotic (5 days)	245 ± 44

Table 20 shows the mean phospholipid content of the liver-plasma pool in nephrotic and control animals five days after injection of rabbit serum. By liver-plasma pool one means the sum of the total liver and plasma phospholipids. Its physiologic meaning stems from the fact that the liver has been shown to be the sole source of phospholipids in this pool, as mentioned before, and that, in untreated rats liver and plasma phospholipids are in rapid equilibrium with each other[16]. Plasma volume determinations were not carried out in this study but comparison of hematocrit values in nephrotic and control animals and data reported by Harris[19] and Craig[20] warrant the assumption of a practically unchanged plasma volume in the nephrotic state. Hence plasma phospholipid content has been calculated on the basis of a plasma volume estimated at 5 per cent of body weight (volume)[21]. The phospholipid pool size is somewhat larger in nephrotic than in control rats, although by less than two standard deviations. The total liver mass, both in terms of wet and dry weight, was increased in nephrotic animals. If one postulates a rapid equilibration of newly synthesized phospholipids between liver and plasma of nephrotic animals, then these values - for unchanged means incorporation and for increased mean pool-size - would suggest a moderate though not statistically significant increment in liver phospholipid synthesis in the nephrotic state.

[18] Drabkin, D. L., and Marsh, J. G., Proceedings of the 7th Annual Conference on the Nephrotic Syndrome, N.Y., pp. 25-38, 1956.

[19] Harris, A. W., and Gibson, J. G., 2nd, Clinical studies on the blood volume in Bright's disease with and without edema, renal insufficiency and congestive heart failure, and in hypertension. J. Clin. Invest. 18: 527, 1939.

[20] Craig, A. B. and Waterhouse, C., The volume of distribution of high molecular weight dextran and its relationship to plasma volume in the human. J. Lab. and Clin. Med. 49: 165, 1957.

[21] Bond, C. F., The nature of the anemia of pregnancy in the rat. Endocrinology 43: 180, 1948.

* The cholesterol liver-plasma pool increase found in the nephrotic rat may well account for the "decrease" in incorporation found here.

However, this interpretation is open to criticism since the rapid liver-plasma equilibrium characteristic of the normal animal is probably impaired in the nephrotic state. Thus, in vivo studies of phosphate phosphorus incorporation into liver and plasma phospholipid phosphorus 24 hours after NTS and 2½ hours after P32 injection reveal in many instances an increase of the ratio of liver phospholipid specific activity to plasma phospholipid specific activity from 1 (in control animals) to 1.6 and even 2 (in nephrotic animals). This suggests impairment of either release of newly synthesized phospholipids into plasma or, more likely, an increment of the plasma-phospholipid pool in excess of the concomitant liver phospholipid pool increment. Such an interpretation would be compatible with an impairment of the re-entry of phospholipids from the plasma into liver cells and/or a decreased phospholipid catabolism.

It is possible to estimate the increment of phospholipid synthesis necessary to bring about the observed mean increase in pool size, if the fractional catabolic rate remained unchanged. The catabolic rate of phospholipid, which in the steady state equals its turnover rate, is presumed to be a first order reaction and has been estimated to be about 5 per cent of the pool in the normal rat[22].

From the data referred to it can be estimated that under these conditions - that is, if an increase in synthetic rate, and only an increase in synthetic rate, were responsible for the hyperlipemia observed in nephrotic rats on the 5th day of their disease - the increment of synthetic rate would have to be about 30 per cent in order to account for the observed increase in pool size. The fact that the observed incorporation data suggest no such increment indicates the presence of a decrease of catabolic rate, which would then be responsible for at least part of the increase in phospholipid pool size. However, an increase of the synthetic rate cannot be ruled out on the basis of these data alone. Indeed, if the amount catabolized per unit time were to remain constant during the development of the syndrome an increase of the synthetic rate of approximately 10 per cent would already suffice to account for the observed changes. Incorporation data usually show standard deviations in that order of magnitude. Because of the wide standard deviations of the mean pool size values one is not fully justified in deriving the conclusion that the phospholipid breakdown rate must be impaired, since this conclusion is based on the mean values only. Nevertheless, the results are entirely compatible with such an interpretation which is further supported by the plasma/liver phospholipid specific activity ratios discussed before. Finally the estimation of the phospholipid turnover in patients with the nephrotic syndrome from phosphate phosphorus into plasma phospholipids by Moser and Emerson[23] would likewise be in agreement with this concept.

Since endogenously labelled phospholipids needed for the study of breakdown rates are difficult to obtain quantitatively in an unaltered condition and in view of the observation of a relatively greater plasma phospholipid pool in nephrotic animals we turned

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[22] Bollman, J. L., Flock, E. V. and Berkson, J., Turnover rate of phospholipid phosphorus by the liver of the white rat. Proc. Soc. Exp. Biol. and Med. 67: 308, 1948.

[23] Moser, H. W., and Emerson, K. Jr., Estimation of the phospholipid phosphorus turnover time in man: Studies in normal individuals, in patients with the nephrotic syndrome and in other types of hyperlipemia. J. Clin. Inv. 34: 1286, 1955.

"Normal" | Hx | Ax

Control

Nephrotic

Phospholipids mg % x 10
Cholesterol mg % x 10
Total Protein gm %

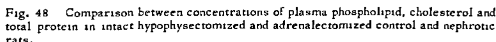


Fig. 48 Comparison between concentrations of plasma phospholipid, cholesterol and total protein in intact hypophysectomized and adrenalectomized control and nephrotic rats.

RAT SERUM
PHOSPHOLIPID VS TOTAL PROTEIN CONCENTRATION

PL mg %

C = Control animals
C-NE = Nephrotic controls (5 d)
HX C = Hypophysectomized controls
HX-NE = Hypoxed-nephrotics (5d)

C-NE (20)
HX-NE on CORTISONE (5)
HX-NE (10/7)
CONTROLS (10)
HX-C (7)

500, 400, 300, 200, 100
1, 2, 3, 4, 5, 6, 7 TP gm %

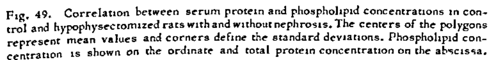


Fig. 49. Correlation between serum protein and phospholipid concentrations in control and hypophysectomized rats with and without nephrosis. The centers of the polygons represent mean values and corners define the standard deviations. Phospholipid concentration is shown on the ordinate and total protein concentration on the abscissa.

from the study of phospholipid synthesis to an investigation of lipid mobilization in the nephrotic state.

This second study* seemed particularly indicated in view of a number of recent reports in the literature. Seifter and Baeder disclosed the isolation of a "lipid mobilizing factor" from the plasma of intact fasting animals which had been given cortisone, failure to find this substance in similarly treated but hypophysectomized animals and presence of the factor in plasma of nephrotic rats[24, 25]. Furthermore Rosenman, Friedman and Byers have suggested that the hyperlipemia observed in the nephrotic state is a direct consequence of the lowered plasma albumin concentration in this condition[26, 27].

In the present study serum concentration of total protein and albumin have been correlated with the degree of hyperlipemia in nephrotic, nephrotic-hypophysectomized and nephrotic-adrenalectomized rats.

Although the food intake of the animals could be controlled within reasonable limits some of the differences in lipid levels which will be discussed may have to be ascribed - at least partly - to small variations in the intake of the experimental groups. In Figure 48 the phospholipid, cholesterol and total protein concentrations of control, control-hypophysectomized and control-adrenalectomized rats are compared with the same parameters in nephrotic, nephrotic-hypophysectomized and nephrotic-adrenalectomized rats. It can be seen that the concentration of the two lipids and of the total protein did not differ widely within the non-nephrotic group whereas in the nephrotic animals cholesterol and phospholipid concentrations were greatly increased. In the nephrotic-hypophysectomized animals, however, the cholesterol and phospholipid levels were considerably less abnormal than in the nephrotic group, and the cholesterol and phospholipid levels remained almost within normal limits in the nephrotic-adrenalectomized group. All three categories of animals exhibited low plasma protein concentrations and severe proteinuria. I will come to this point a little later. It should also be mentioned that none of these differences could be attributed to variations in the degree of hemoconcentration in the three nephrotic groups.

The isolation of a lipid mobilizing factor similar to that described by Seifter from nephrotic rats at the stage of maximal edema and hyperlipemia has been attempted. Two normal rats were injected intravenously with the equivalent of 40 cc. of nephrotic

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[24] Seifter, J. and Baeder, D. H., Lipemia clearing by hyaluronidase and its inhibition by cortisone, stress and nephrosis. Proc. Soc. Exp. Biol. Med. 86: 709, 1954.
[25] Seifter, J. and Baeder, D. H., Occurrence in plasma of an extractable lipid mobilizer (LM). Proc. Soc. Exp. Biol. Med. 91: 42, 1956.
[26] Rosenman, R. H., Friedman, M., and Byers, S. O., The causal role of plasma albumin deficiency in experimental nephrotic hyperlipemia and hypercholesterolemia. J. Clin. Invest. 35: 522, 1956.
[27] Rosenman, H. R., and Friedman, M., In vivo studies on the role of albumin in endogenous and heparin activated lipemia clearing in nephrotic rats. J. Clin. Invest. 36: 700, 1957.

*This study was made possible through a U. S. Public Health Grant No. H-444 (C7) and a grant from the Massachusetts Heart Association.

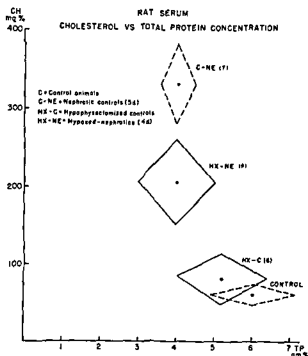


Fig. 50· Correlation of serum cholesterol with serum protein levels in control and hypophysectomized, nephrotic and non-nephrotic rats. The polygons have the same meaning as in Fig. 49.

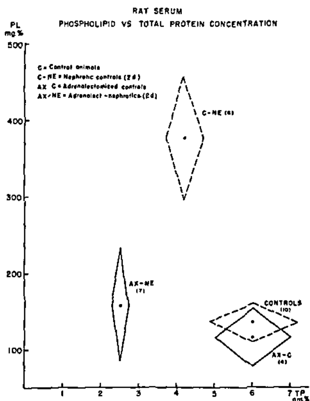


Fig. 51 Illustrates the effect of adrenalectomy upon the correlation between phospholipid and total protein concentration in nephrotic and non-nephrotic animals. The central dot represents mean values and the polygon indicates standard deviation.

rat ascites (redissolved, lyophilized dialysate) and similarly with the equivalent of 40 cc. of human nephrotic serum. A hyperlipemic response in these test rats was not observed. The test rats presumably had normal liver function.

Also, cortisone given for two days prior to sacrifice (.1 mg/100 g/day, i.m.) resulted in a partial restoration of the hyperlipemic response to the nephrotoxic serum in hypophysectomized and adrenalectomized rats. (Fig. 49 dotted line.) These results do not support the hypothesis that the dialysable lipid mobilizing factor described by Seifter and Baeder is responsible for the increased lipid mobilization observed in the nephrotic state produced in these animals.

In Figure 49 serum protein levels have been correlated with serum phospholipid concentrations in control and hypophysectomized rats with and without nephrosis (5 days after NTS injection). The centers of the polygons represent the mean values, the corners the standard deviations. Similarly, in Figure 50 serum protein levels have been correlated with serum cholesterol concentrations. It is evident that the reciprocal correlation seen in the nephrotic animals, when compared with the control animals, does not hold in the nephrotic-hypophysectomized animals, which show proportionally less hyperlipemia.

DR. HEYMANN: *What is the ordinate?*

DR. BALLY: The ordinate represents phospholipids in mg. per cent and the abscissa is total proteins in grams per cent.

The next Figures (51, 52) show the same correlation as in Figures 49 and 50, but in this instance the effect of adrenalectomy was under study. It is apparent that in the nephrotic-adrenalectomized animals (3 days after injection of NTS), the hyperlipemic response (with respect to both cholesterol and phospholipid concentrations) was greatly reduced, even though the total protein concentration was lower than in the nephrotic animals sacrificed at the same interval after NTS injection.

Determination of rat albumin in the lower concentration range caused considerable difficulty because of the concomitant rise in the alpha-1-globulin fraction. The alpha-1-globulin fraction carries a considerable amount of low density lipoproteins in sera from nephrotic rats. The albumin data reported below have been obtained with zone electrophoresis on Whatman No. 3 paper for higher albumin concentrations and on thin paper (Schleicher and Schuell), as well as by the dye method of Rutstein and Ingenido[28] for low albumin concentrations. Although the total protein to albumin correlations are not entirely linear, it would appear that a reasonable approximation of the albumin to lipid relationship in the sera of these animals is obtained by plotting total protein concentration against lipid concentration.

Thus, when, as shown in Figure 53, lipid concentrations have been plotted against estimated albumin concentrations, essentially the same relationship was demonstrated as in the previous figures. Nephrotic hypophysectomized rats show a somewhat less

[28] Rutstein, D. D., Ingenido, E. F. and Reynolds, W. E., The determination of albumin in human blood plasma and serum. J. Clin. Inv. 33: 211, 1954.

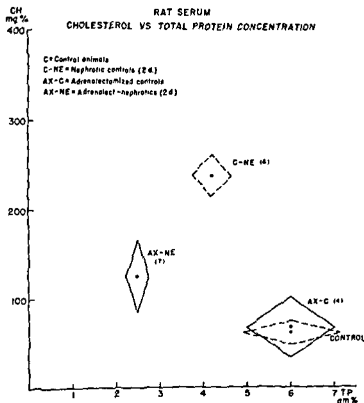


Fig. 52 Correlation between cholesterol and total protein concentration in adrenalectomized nephrotic and non-nephrotic rats.

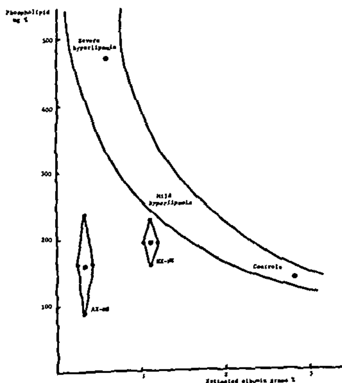


Fig. 53· Correlation between serum lipid concentration and estimated albumin con- [illegible] total protein determination [illegible] the latitude of phospho- [illegible] stages of hyperlipemia.

marked hyperlipemia than would be expected from their serum albumin concentration and strikingly lower than expected serum lipid concentrations are evident in the nephrotic adrenalectomized group.

These measurements demonstrate the absence of a rigorous or even close correlation between albumin concentration on the one hand and cholesterol and phospholipid concentrations on the other hand in rats without pituitary or adrenal glands. Such a correlation would be expected if a defective "clearing mechanism" were solely responsible for the accumulation of low density materials. They imply at least some contribution of lipid mobilization from depots in bringing about the final hyperlipemia. In this connection I would like to mention parenthetically that a preliminary study performed in collaboration with Dr. Albert Winegrad failed to show impairment of lipogenesis in nephrotic rats from glucose by adipose tissue incubated in vitro. It would appear that, at least in this respect, the accumulation of serum lipids in the nephrotic state bears no resemblance to the hyperlipemia of diabetic ketosis and bears out our opinion that to date no clear-cut evidence for impaired carbohydrate metabolism in the nephrotic syndrome has been forthcoming.

This failure to observe a correlation between albumin concentrations and nephrotic hyperlipemia after endocrine manipulation cast some doubt upon the postulated exclusive causal relationship between hypoalbuminemia and hyperlipemia in the nephrotic syndrome led to the third study* which I would like to report. It was again concerned with lipid-albumin relationships and was carried out in collaboration with Dr. Shosaku Numa of the Department of Physical Chemistry, Harvard Medical School, and with Dr. K. Ott of the Department of Medicine, Tuebingen, Germany, (Director: Professor H. Bennhold).

Professor Bennhold in 1954 had observed and described two patients, brother (25 years) and sister (33 years), demonstrating total absence of albumin in their serum [29, 30].

Dr. Ott has informed me that 150 other members of the family who could be tested had normal serum albumin concentrations. It is interesting that this woman, the patient whose serum we fractionated, presented herself four years ago to a general practitioner in Southern Germany, because after a heavy day's work in the fields, she noticed a little ankle edema. Otherwise she felt well. The physician found a markedly accelerated sedimentation rate and referred the patient to Dr. Bennhold's clinic where a serum electrophoresis and immunochemical studies revealed the total absence of albumin. After a considerable number of studies had been carried out, she received an intravenous infusion of albumin in order to study albumin degradation, which was found greatly decreased. The patient felt "better" after the infusion.

The studies reported today were done almost a year after this infusion. An immunochemical determination which Dr. Gitlin and Dr. Gross were kind enough to

[29] Bennhold, H., Peters, H. und Roth, E., In: Verhandlungen der Deut. Ges. Innere Med., Munich, 1954.
[30] Ott, H., Das Blutserum bei Analbuminemie, Z. ges. Exp. Med. 128. 340, 1957.

*This study was made possible through a U. S. Public Health Grant H-444 (C7) and a grant from the Massachusetts Heart Association.

FRACTIONATION OF RAT SERUM

β LP

NEPHROTIC SYNDROME

Normal (C) & Hypox Rats (Hx)

x● = Cholesterol approx. mg %

▼■ = Phospholipids approx. mg %

[ϱ Range 1021 – 1063]

Section No.	Chol/PL Hx	Chol/PL C
1	1 01	.45
2	2.54	.55
3	1 49	1 25
4	2.2	1 55
5	1 66	1 45
6	1 62	1 43
7	1 42	1.35
8-9		1 08
8-10	1 22	

Abscissa: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100

Fig. 54 Fractionation of rat serum indicating the beta lipoprotein distribution in terms of cholesterol and phospholipid concentrations in pooled sera of intact and hypophysectomized nephrotic rats The section number on the ordinate represents a density range between 1 (1.021) and 8-10 (1.063). Concentration of cholesterol and phospholipids are presented on the abscissa in milligrams per cent. Most of the increase in cholesterol and phospholipid concentration is found in the high density beta lipoproteins, section 3-6.

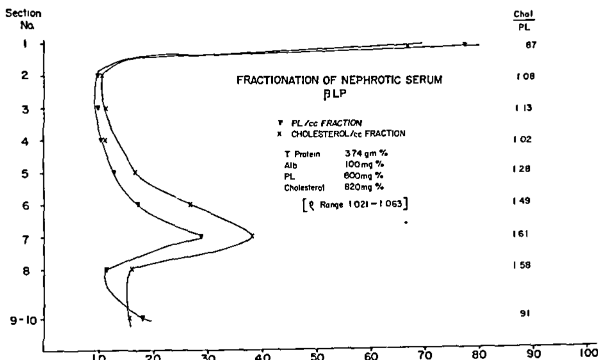


Fig. 55: Lipoprotein fractionation of serum from a nephrotic patient having a serum albumin concentration of 100 mg.%. The highest concentrations of phospholipid and cholesterol occur in the low density fraction.

perform, showed an albumin concentration between 35 and 40 mg. per cent. Both this patient and her affected brother have a moderate hyperlipemia. The patient's total protein was 4.6 gm. per cent, serum cholesterol was 304 mg. per cent and serum phospholipids were 427 mg. per cent. The serum was clear.

All frationation data reported here have been obtained with the method of Oncley and Mannick[31] in a gradient density tube. The procedure followed was essentially the same as that described by Dr. Rodbell at this symposium. The only difference was that, instead of spinning lipoproteans in different tubes, each with a uniform given density, one uses a single tube with a density gradient, i.e. a tube in which the density varies with the distance from the bottom.

The limits here have been chosen so as to encompass the beta lipoprotein densities (1.021 to 1.063 at the end of an experiment). This is a convenient means of obtaining a spectrum of the beta lipoproteins in one - or rather two - centrifugations. The following illustrations show a comparison of the beta lipoprotein distribution over this gradient in terms of total protein, cholesterol and phospholipid concentrations in a pool of serum from nephrotic and nephrotic-hypophysectomized rats, in the serum of a nephrotic patient and in the serum of Dr. Bennhold's analbuminemic patient. Figure 54 represents the fractionation data in the nephrotic rat. Each section on the ordinate represents a small density range. The top section around the density of 1.021, the fifth section around 1.035, the bottom section around 1.063. On the abscissa the concentrations of the different serum constituents determined are given in mg. per cent or g. per cent. It can be seen that in nephrotic rats a very large share of the hyperlipemia is carried in the low density range of the beta lipoproteins. (The term "beta" here refers to the nomenclature of Oncley[32] and does not necessarily coincide with the nomenclature derived from electrophoresis at pH 8.6.) The serum of nephrotic-hypophysectomized rats shows a pattern similar to what one might find in a serum from a patient with very mild nephrosis and with only moderately depressed albumin concentration: most of the increase in cholesterol and phospholipid concentration is found in the high density lipoproteins (section 3-6). Figure 55 shows a serum beta lipoprotein fractionation from a highly nephrotic patient. The serum albumin concentration was 100 mg.% as determined immunochemically. The beta low density fraction is very high. The high density beta fraction is also elevated but comparatively less so. In comparison with this I now would like to show you the data obtained on the "analbuminemic" patient's serum (Fig. 56). I should point out that the magnitude of the plotted concentrations can only be compared percentage-wise, that is, with respect to the other concentrations in the same fractionation run, since different amounts of serum have been used for different runs and these figures have not been standardized against each other.

The serum was fractionated only two days after it had been withdrawn in Tuebingen and was kept at a temperature of zero degrees at all times during the flight (courtesy

[31 Oncley, J.L. and Mannick, V.G., Sedimentation Analysis of plasma proteins, p. 1. Am. Chem. Soc. 126th Meeting, N.Y., 1954.

[32] Oncley, J. L., Gurd, F. R. N. and Melin, N., Preparation and properties of serum and plasma proteins. xxv Composition and properties of human serum beta lipoproteins, J. Am. Chem. Soc. 72 458, 1950.

Section No.

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x • = Cholesterol approx. mg %

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[ρ Range 1.021 – 1.063]

Section No.	Chol/PL Hx	Chol/PL C
1	1.01	.45
2	2.54	.55
3	1.49	1.25
4	2.2	1.55
5	1.66	1.45
6	1.62	1.43
7	1.42	1.35
8-9		1.08
8-10	1.22	

10 20 30 40 50 60 70 80 90 100

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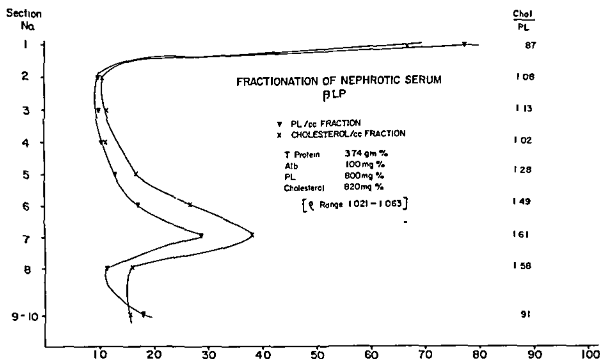


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8-10	1.22	

10 20 30 40 50 60 70 80 90 100

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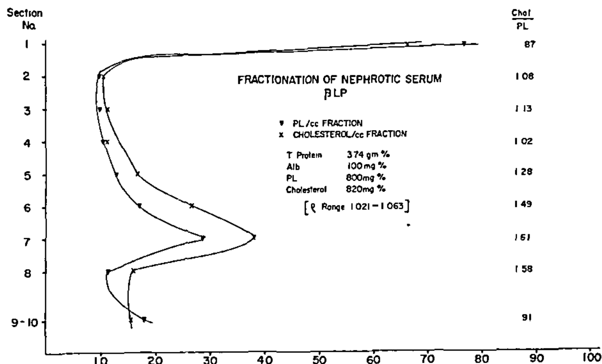


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[32] Oncley, J. L., Gurd, F. R. N. and Melin, N., Preparation and properties of serum and plasma proteins. xxv. Composition and properties of human serum beta lipoproteins. J. Am. Chem. Soc. 72. 458, 1950.

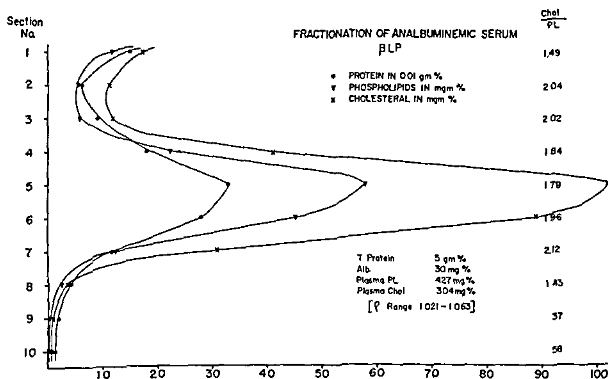


Fig. 56: Distribution of phospholipids and cholesterol in the beta lipoprotein moieties derived from a patient who presumably had congenital abscence of serum albumin.

Dr. Renold). EDTA had been added as a protective agent (0.05%). This patient does not present a hyperlipemia nearly as severe as one might expect in a nephrotic patient with comparatively severe hypoalbuminemia. The serum was clear, as previously mentioned. The distribution in the density gradient tube further shows that - in contrast with the nephrotic patient's serum - very little elevation if any is found in the low density beta range. (All sera fractionated were obtained in the fasting state and chylomicra were not previously removed, they are included in the top fraction. They and the low density beta lipoproteins are referred to as low density material.) There is considerable elevation in the high density beta fraction. Indeed, in terms of the total serum lipids, the amount of lipid present in this fraction accounts for about 80 per cent of the total cholesterol and 50 to 60 per cent of the phospholipids.

Since one would expect the low density material to accumulate if diminished "clearing activity" were responsible for the hyperlipemia observed here, presumably due to a low concentration of unesterified fatty acid acceptor, i.e. albumin, these data again do not support the hypothesis that impaired clearing activity per se and alone is responsible for the elevated lipid concentrations observed in hypoalbuminemia states.

CHAIRMAN GITLIN: Is there any discussion of this report?

DR. HEYMANN: I wonder whether Dr. Bally takes or does not take issue with some results that we obtained in 1953[33] relative to the effect of hypophysectomy or adrenalectomy on blood lipid concentrations.

Figure 57 shows on the left hypophysectomized and on the right control rats injected with the same dose of the same batch of anti-kidney serum. It can be seen that the hypophysectomized group developed a milder disease. They had [illegible] uria, less, if any, edema and ascites, and less [illegible] ar results obtained in adrenalectomized rats [illegible] nephrotic renal disease had persisted for [illegible] (Column A), or when it had been performed 10 to 21 days prior to the injection of anti-kidney serum (Column B) proteinuria and degree of anasarca were lessened in the operated animals.

The average values of blood lipids obtained in these experiments are listed in Table 21. The degree of hyperlipemia was less marked in the hypophysectomized and adrenalectomized nephrotic animals than in rats that had not been operated but had

TABLE 21

EFFECT OF HYPOPHYSECTOMY OR ADRENALECTOMY ON SERUM CHOLESTEROL

	Serum Cholesterol mg.%	Serum Total Lipids mg.%
Healthy Control Rats	90	400
Hypophysectomized Nephrotic Rats	254	755
Adrenalectomized Nephrotic Rats	200	894

[33] Heymann, W., Hackel, D. B., Gilkey, C. and Salehar, M., Relation of Pituitary and Adrenal Glands to the Nephrotic Syndrome in Rats. Lab. Invest., 2. 423, 1953.

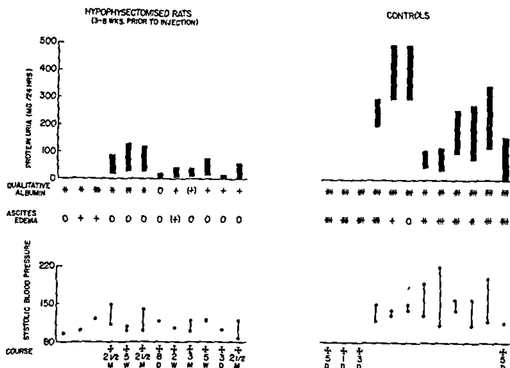


Fig. 57. Comparison of the intensity of the nephrotic syndrome occurring in hypophysectomized versus intact rats made nephrotic with nephrotoxic serum. Hypophysectomized animals developed milder disease.

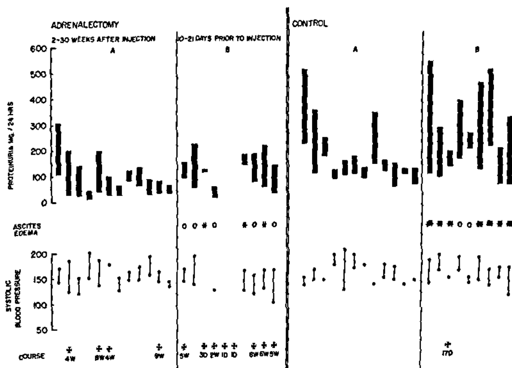


Fig. 58. Comparison between severity of the nephrotic syndrome in rats subjected to adrenalectomy before or after injection of nephrotoxic serum related to intact nephrotic animals.

been injected with the same dose of the same nephrotoxic serum. Nevertheless, the hyperlipemia in these animals certainly was still present, representing a quite remarkable degree of nephrotic hyperlipemia. Considering that the disease was less intensive in other manifestations as well as, for instance, proteinuria and edema formation, a specific influence of the removal of the pituitaries or of the adrenals on blood lipid concentrations cannot be assumed. The values are in line with the severity of the resulting disease. I wonder, Dr. Bally, whether your studies have led you to different conclusions?

DR. BALLY: No, I think I agree with Dr. Heymann in that the response is lessened but still there; and that, of course, nutritional factors and factors of metabolic requirements enter the picture; factors which are unfortunately excluded only with great difficulty.

I purposely did not report any urinary protein losses, although all rats had severe proteinuria, since the overall catabolic and excretory losses do not necessarily correlate with the urinary losses alone. I simply related the serum lipid concentration to the serum albumin or total protein concentration. I think it is in terms of the correlation that the data on nephrotic-hypophysectomized and nephrotic-adrenalectomized rats attain their significance. The nephrotic-hypophysectomized rat shows a lesser deviation from the nephrotic animal than the nephrotic-adrenalectomized rat. The latter develops severe edema - presumably in the absence of aldosterone - even more so than the control-nephrotic rat. Proteinuria and depression of serum protein and serum albumin concentrations were just as severe as in the intact nephrotic animals or even more severe in the case of the nephrotic-adrenalectomized group. I know of no well-defined lipid mobilizing action of the corticosteroids by themselves.

We are now studying the effect of adrenal demedullation on the hyperlipemia of the nephrotic state. I am not, at the present time, ready to interpret these data in terms of a well-defined endocrine disturbance.

DR. HEYMANN: May I ask you whether the hyperlipemia which you obtained in these severely nephrotic adrenalectomized rats was very marked?

DR. BALLY: The lipid concentration was low.

DR. HEYMANN: Do you remember the total lipid values?

Dr. BALLY: I do not have any total lipid values available. The phospholipid values were in the neighborhood of 140 mg.%.

DR. HEYMANN: Phospholipid concentrations are usually not as markedly increased. They are usually less increased as are cholesterol and fatty acid values.

DR. BALLY: I thought the cholesterol concentration was also markedly lower than expected. I would think that if one were to do a t-test on the cholesterol values in Figure 52 one would find a significant difference at the p less than .1 level.

CHAIRMAN GITLIN: Dr. Gordon, would you like to make a comment? Apparently, there is one other analbuminemic patient in the world, and Dr. Gordon has control over that one.

DR. GORDON: No, I do not control the analbuminemic subject. She is really a patient of Dr. Frederick Bartter, but he has been kind enough to allow me to make some observations on her. I have corresponded with Bennhold; and I think these criteria are fulfilled by all three patients. First, the primary defect seems to be the failure to synthesize albumin. This can be shown by the fact that these people have very low albumin in the steady state, but, if albumin is given, it is degraded very slowly. Rather extensive medical work-up indicates no disturbance of hepatic, renal, or other measurable functions in these subjects. Second, when their serum albumins are allowed to fall to low levels, they develop a serum lipid pattern which is similar to Class 3 nephrotics, in the terminology used by Dr. Baxter.

The elegant slide which Dr. Bally just showed has more data than we have ever accumulated on our subject but when the serum albumin of our patient is allowed to fall below 1 g. per cent. she develops a total serum cholesterol of somewhat over 400 mg. per cent, a total serum phospholipid slightly over 300, and a normal triglyceride, a pattern which is entirely consistent with an elevation of the Sf 3-8 beta lipoprotein. In every one of these three cases, the serum is always clear, and never assumes the highly lactescent appearance one finds in nephrosis.

We have treated the patient at NIH with large amounts of human albumin which, fortunately, is easily available. Let me draw you a graph of the changes we observed in the serum lipids, when albumin was given at the rate of 50 g. a day for 12 days. The patient's serum albumin had fallen to about 0.7 gm. per cent, and of course it rapidly rose during the period of infusion. Subsequent disappearance was very slow. Total serum cholesterol was about 400 mg.% before the infusion of albumin, and dropped to 130 mg.% afterward.

This patient has never been studied extensively at a time when no albumin had been given. Unfortunately for us and fortunately for her, albumin was given to her years ago; and we have never been patient enough to wait long enough for her level to drop to zero. In particular, she develops mild to moderate edema and an annoying lassitude when depleted of albumin, so that she is unwilling to go indefinitely without treatment. In addition, we are concerned that the greatly elevated serum cholesterol might predispose her to the development of coronary artery disease.

The disappearance of the passively infused albumin proceeds with a half-time of approximately 56 days, which is almost three times as long as the half-time for disappearance of serum albumin as measured with I^{131}-labelled albumin in normal subjects. Her deficiency clearly is not due to excessive degradation of albumin.

DR. HEYMANN: Her phospholipids were increased also?

DR. GORDON: Yes, if you plot phospholipids on the same scale as cholesterol, the serum starts at the same level and decreases somewhat less after the albumin infusion. Finally, if you follow the ratio of cholesterol to phospholipid, it tends to decrease, changing from a ratio characteristic of the Sf 3-8 lipoprotein down to one which is normal for human serum.

DR. HEYMANN: Let me just state that this would be most unusual in a nephrotic patient. We always find an increase of all lipid fractions. The fatty acids are in fact usually more markedly increased than cholesterol and phospholipid concentrations.

DR. LANGE: Was this woman very edematous?

DR. GORDON: Very slightly.

DR. LANGE: This is just another tombstone on the grave of the theory of hypoalbuminemia as the most important cause of edema formation in the nephrotic syndrome.

DR. GORDON: We have studied her unesterified fatty acids only to a limited extent. We had not yet worked out a good analytical method when the albumin withdrawal experiment was carried out, so the data are somewhat questionable. Her serum unesterified fatty acids were approximately half of normal, so that the molar ratio of UFA to albumin was elevated. All of these variables assumed normal values after albumin administration.

DR. GITLIN: What was the plasma oncotic pressure in Bennhold's case?

DR. BALLY: It was low; I do not have it here.

CHAIRMAN GITLIN: Why don't we discuss that later?

DR. BALLY: May I ask one question? Have you ever given an infusion of unesterified fatty acid in any form, to see whether they behave normally in these analbuminemic patients?

DR. GORDON: We cannot give a large quantity of unesterified fatty acids. What we can give is a tracer; but we have not done that experiment in this patient.

DR. BALLY: Do you have any idea of what the distribution would be?

DR. GORDON: No.

CHAIRMAN GITLIN. Wait a moment, everyone; Dr. Seifter has some remarks to make. So, Dr. Seifter!

DR. JOSEPH SEIFTER (Philadelphia, Pennsylvania): Thank you.

Several years ago, we had occasion to demonstrate that the administration of cortisone, or subjecting animals to stress, or rendering rats nephrotic, resulted in the release of an inhibitor to the heparin clearing factor which could be demonstrated in the plasma of these animals. The fact that cortisone and that stress cause release of such a factor has been confirmed by others. No one has done work with anti-kidney serum.

The occurrence of such an inhibitor in human disease states also recently has been reported by Levy.

We demonstrated experimentally also that this inhibitor is probably released from the pituitary. Protamine, which is considered to be a specific antagonist to heparin, produces hyperlipemia presumably by inactivating the clearing factor in vivo.

Therefore, we injected protamine into intact rats, and found that in the adrenalectomized rats, protamine was no longer active.

A similar observation has recently been reported by Schatz and Page. Presumably, the hyperlipemia produced by protamine is another stress or intoxication phenomenon.

Contrary to what Dr. Bally said, we have never reported that the lipid mobilizing factor (LM) was inactive in the hypophysectomized animals; in fact, we reported just the opposite.

DR. BALLY: I said cortisone was said not to elicit the hyperlipemic response in the hypophysectomized rat. I was not talking about the lipid mobilizing factor. I am sorry, I just meant to straighten that out.

DR. SEIFTER: Because of the difficulty in eliciting the hyperlipemic effect, and because of the induced endocrine disorders in our animals, we had to consider the role of the liver in the release of lipids or production of hyperlipemia.

We injected normal dogs with the lipid-mobilizing factor, and collected blood from superior mesenteric veins and from the vena cava, by catheterization. This is prehepatic blood. The injection of the mobilizing factor produced marked elevation of lipids in the prehepatic blood which consisted of the triglycerides or total fatty acid exclusively, whereas the posthepatic blood coming from the liver was not hyperlipemic.

Obviously the liver played a role in lipid clearing.

In animals which had been sensitized by the administration of a small amount of an hepatic substance, the injection of the lipid-mobilizing factor produced a marked elevation of triglycerides in mesenteric vein blood; there was an apparent hyperlipemia in the posthepatic circulation. Under these circumstances the liver had added cholesterol and phospholipid to the circulation.

We then performed similar studies in patients at operation. For example, in a patient undergoing a sigmoid resection, samples were taken at the beginning of the operation and prehepatic and posthepatic blood were clear. Two hours after the operation was completed, there was a tremendous lactescent plasma coming to the liver; but, the blood leaving the liver was clear. By chemical analysis, this lactescence was due to the increase of total fatty acids almost exclusively.

The liver, here again, has taken out the mobilized lipid and therefore decreased its concentration in the posthepatic blood.

In another patient, undergoing the splenectomy, the blood coming to the liver from the mesenteric bed picked up a tremendous amount of neutral fat, which was disposed of somehow by the liver.

In a patient who had a cholecystectomy, at the end of the operation, the blood coming to the liver had a normal cholesterol content, but a considerable elevation of the total fatty acid. The blood leaving the liver had a higher concentration of cholesterol, and perhaps, had lost some neutral fat.

We present these data to show that peripheral hyperlipemia is a rather complicated phenomenon involving not only the pituitary but also an element of stress (particularly in the first 72 hours in nephrotic rats), all in addition to the role of the liver, and probably other factors.

Thank you.

CHAIRMAN GITLIN: Thank you, Dr. Seifter.

The meeting will adjourn to reconvene after lunch.

(The meeting adjourned at one-twenty o'clock.)

III. ELECTROLYTE METABOLISM

The meeting reconvened at two-thirty o'clock, Dr. Metcoff presiding.

CHAIRMAN METCOFF: This afternoon's session is on electrolyte metabolism. I have reluctantly agreed to be chairman.

The first speaker will be Dr. Orloff who will talk on hyponatremia. Jack!

A. Hyponatremia

DR. JACK ORLOFF (National Heart Institute): I feel rather hesitant to talk about this subject before this group, since I am reasonably certain that many of you have had a great deal more clinical experience with the syndrome than we have had here.

What I plan to do is to summarize briefly some of our present ideas concerning the pathogenesis of hyponatremia, and illustrate one or more points with a few specific examples taken from our wards.

As all of you know, sodium together with its accompanying anions make up approximately 90 per cent of the osmotically active constituents of the extracellular fluid. Therefore the finding of a low concentration of sodium in plasma is always indicative, with two important clinical exceptions, of a low-effective osmotic pressure of body fluids.

Consequently hyponatremia must be secondary to the relative retention of water in excess of solute in the body. A low plasma sodium is not an indication of body stores of sodium and in fact, dilution hyponatremia is commonly observed in edematous patients in whom the total amount of sodium in the body is undoubtedly increased.

The two exceptions to the generalization that hyponatremia is synonymous with hypo-osmolality (a low effective osmotic pressure) are hyperglycemia and hyperlipemia.

In the first instance (hyperglycemia), the equilibrium effective osmotic pressure may be normal or elevated; but the concentration of sodium in plasma is depressed by virtue of a shift of water out of cells and expansion of the extracellular-fluid compartment engendered by the addition of a relatively non-penetrable, osmotically active solute (glucose).

Hyponatremia in hyperlipemia is due to a reduction in the water content of a unit of plasma, due to displacement of water by the large lipid molecules. Consequently, though one will observe a low concentration of sodium in a milliliter of plasma, the concentration of sodium in a milliliter of plasma water is normal, as is the effective osmotic pressure.

Since, exclusive of the two exceptions which I have just mentioned, hyponatremia is always a consequence of water retention, the common denominator in all cases must be an inability to excrete ingested water with sufficient rapidity to maintain a normal osmotic pressure of body fluids.

The question we should like to ask, then, is, why don't these patients excrete water normally? Why are they unable to respond to ingested water in a normal manner, that is, excrete the water, and return the plasma osmotic pressure to normal?

The regulation of water balance in man is sufficiently complex to provide opportunities for a large variety of abnormalities, any one of which may lead to an impairment of water excretion and, resultant hypo-osmolality.

In order to define the numerous defects in regulation which theoretically can produce hypo-osmolality, and to provide a basis for a classification of this disorder I would like to review briefly the important intratubular events which transpire in the process of urinary dilution and concentration.

As you all know the regulation of water balance is dependent mainly on an intact hypothalamic-pituitary system and the kidneys. Approximately 80-85 per cent of the glomerular filtrate is reabsorbed in the proximal segment essentially as an osmotic solution. Presumably sodium and chloride are actively reabsorbed from the urine and water follows passively along the osmotic gradient established by electrolyte abstraction. The remaining isosmotic 15 per cent of urine is delivered to the distal segment where the processes we are primarily concerned with, dilution and concentration, occur.

In the absence of antidiuretic hormone (ADH), the following sequence of events is thought to occur. Sodium and anion are actively reabsorbed from the tubule fluid, leaving water behind. This in essence is the mechanism of urinary dilution. Water freed by solute removal has been termed C_{H_2O} (solute-free water) by Smith.

In the absence of ADH, the membrane in the diluting segment has a peculiar property. It remains relatively impermeable to water, presumably so-called "pores" in the membrane are closed. This provides a barrier to the outward diffusion of freed water and a hypotonic solution is delivered to the terminal segment (collecting duct). Here it is presumed that water without solute is removed.

The important point is that although water is removed in the collecting duct, the volume removed is insufficient to result in the development of either an isotonic or a hypertonic solution and a dilute urine is excreted into the bladder.

In the presence of ADH, the sequence of events differs. Sodium chloride and water are removed in the proximal segment just as in the process of urinary dilution

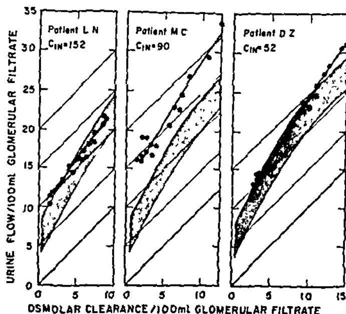


Fig. 59. The effect of combined water and osmotic diuresis on urine flow and solute excretion in patients with the nephrotic syndrome.

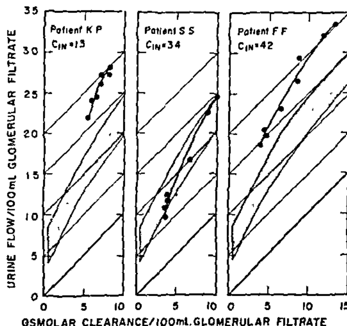


Fig. 60. The effect of combined water and osmotic diuresis on urine flow and solute excretion in patients with the nephrotic syndrome.

and once again a small volume of residual isosmotic urine is delivered to the distal segment. The tubule membrane in this area, however, is now freely permeable to water due to the presence of ADH, and although electrolyte is abstracted, freeing water in the process, the water may now diffuse out of the tubule until osmotic equilibrium is achieved. A small volume of isosmotic fluid is delivered to the collecting system where once again water without solute is removed resulting in the development of a hypertonic urine which may now be excreted.

In view of what I have just said, it should be evident that there are at least four requirements for the rapid excretion of water and the elaboration of a dilute urine. The first of these is adequacy of filtration; secondly, volume flow to the diluting site must be adequate (that is, sufficient substrate (NaCl) must be delivered to the diluting segment), thirdly, the capacity of the reabsorptive system for sodium and anion must be normal, and finally, the membrane in the distal segment must remain virtually impermeable to water. This last is a function of an intact hypothalmic hypophyseal system. I should like to propose that most, if not all, cases of dilution hyponatremia may be considered as being due to a disturbance in one or more of these four factors.

The first disturbance I would like to consider is inadequacy of filtration. This is an obvious cause of hypo-osmolality. It is certainly the situation in the anuric patient and is at least a contributory factor in patients with renal insufficiency with hyponatremia in whom the number of functioning nephrons is reduced. In this group, when filtration rate is seriously depressed and insufficient urine is delivered to the distal segment, even were it operating normally, the relatively small volume of urine which can be excreted may be insufficient to overcome dilution produced by the administration of intravenous fluids, and in some instances hyponatremia may develop on what would otherwise be considered a normal fluid intake, though admittedly an added factor in these patients may well be persistent ADH secretion. Even in normal man it is possible, though certainly a rare experience, to overwhelm the filtering capacity of the kidney and produce water intoxication. On the other hand, and this is not an uncommon experience, if loss of extracellular fluid through vomiting, diarrhea, excessive sweating, etc., compromises glomerular filtration, water administered in order to replace volume losses may not be excreted rapidly enough to prevent hyponatremia. Presumably ADH secretion and resultant urine hypertonicity is a contributory factor in this instance.

In order to determine how important the reduction in number of functioning nephrons is insofar as hyponatremia is concerned, Dr. Walser and I studied a number of patients with renal disease. The response to combined water and osmotic diuresis (mannitol) is shown in Figure 59. Urine flow is plotted as a function of solute excretion or osmolar clearance. The diagonal line represents the excretion of an isotonic urine whereas all points above the line represent the excretion of a dilute urine. The distance between the diagonal line and any point represents the clearance of free water. The shaded area represents the normal response to combined water and solute diuresis. In essence we have used this technique as a means of estimating diluting ability during suppression of ADH secretion. In order to compare patients with different filtration rates, the data have been expressed as urine flow and solute excretion per 100 ml. of GFR. It is presumed that this indicates diluting capacity per nephron unit. It is apparent that all three of these patients (nephrotics) were able to dilute normally despite marked

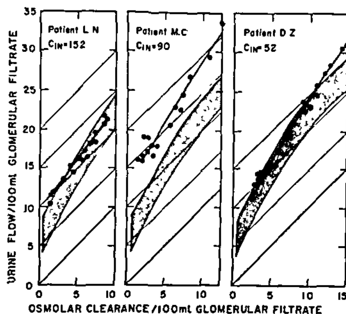


Fig. 59. The effect of combined water and osmotic diuresis on urine flow and solute excretion in patients with the nephrotic syndrome.

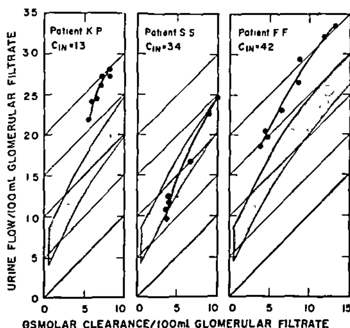


Fig. 60. The effect of combined water and osmotic diuresis on urine flow and solute excretion in patients with the nephrotic syndrome.

and once again a small volume of residual isosmotic urine is delivered to the distal segment. The tubule membrane in this area, however, is now freely permeable to water due to the presence of ADH, and although electrolyte is abstracted, freeing water in the process, the water may now diffuse out of the tubule until osmotic equilibrium is achieved. A small volume of isosmotic fluid is delivered to the collecting system where once again water without solute is removed resulting in the development of a hypertonic urine which may now be excreted.

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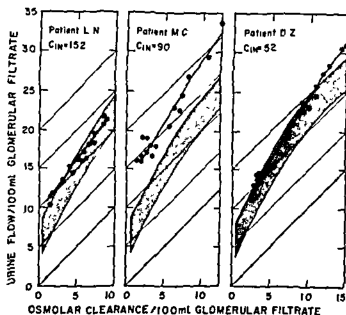


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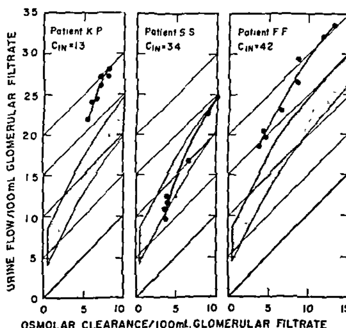


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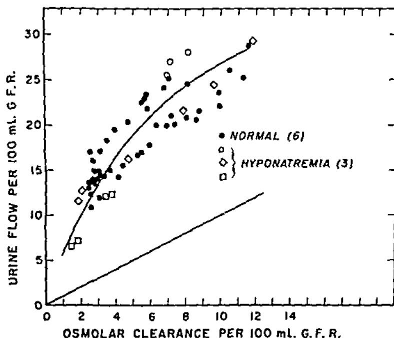


Fig. 61. The effect of combined water and osmotic diuresis on urine flow and solute excretion in normal subjects and in patients with hyponatremia.

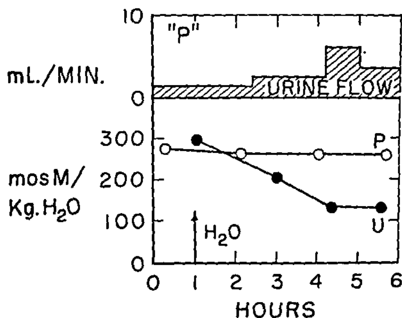


Fig. 62. The effect of water administration on urine flow and urine osmolality in a patient with hyponatremia and renal insufficiency.

differences in GFR. This is also noted in Figure 60. In the first patient, despite an inulin clearance of 13 ml/min. C_{H_2O} clearance per nephron unit was normal. It can be calculated that even in this patient, despite the marked reduction in filtration rate C_{H_2O} is such, that in the absence of ADH it would be unlikely that hyponatremia could develop on a normal fluid intake. In fact, it can be calculated that persistent dilution would only occur were something like 4L a day of pure water administered. We are forced to conclude that even when GFR is reduced, if residual tissue functions normally insofar as dilution is concerned, some additional factor is essential to provide the basis for hyponatremia. By exclusion we have concluded that a change in membrane permeability due to persistent ADH secretion was essential for the development of hyponatremia in these subjects. I don't plan to discuss this in any detail except to say that others as [illegible] secretion of ADH. B [illegible] fall in plasma os [illegible] f the secretory process. Furthermore, in patients such as these, ADH secretion can be interfered with by a further acute reduction in osmotic pressure. For want of a better explanation, this has been ascribed to a resetting of the osmoreceptors or osmostats in the anterior hypothalamus. Figure 61, similarly plotted, demonstrates that in fact an acute water load plus mannitol does interfere with ADH secretion in some hyponatremics. Two of the patients studied were nephrotics with persistent hyponatremia and urine hypertonicity. In these the response was analogous to that observed in normals. In the next slide, Figure 62, it can be seen that urine concentration fell following a water load as would be predicted if the osmostat were set at a lower level.

Dilution hyponatremia may also occur, as already indicated, as a result of a reduction in volume flow to the diluting segment as a consequence of enhanced proximal reabsorption. Dr. Kennedy in our laboratory has studied a few patients of this nature. These were edematous cardiacs in whom the combination of a fall in GFR, and enhanced proximal reabsorption, perhaps characteristic of congestive heart failure, presumably resulted in a marked fall in the volume of isotonic urine entering the distal segment. Under these circumstances, since substrate (NaCl in this instance) is diminished, even were the transport system normal, it would not be possible to produce sufficient Na+ free water in the urine to prevent the development of hyponatremia. Added evidence for this hypothesis were the associated findings of hyperkalemia and acidosis. As you know, Na+ is reabsorbed in exchange for K+ and H+, so that one might expect were insufficient Na+ reabsorbed, neither K+ nor H+ could be excreted efficiently and retention of these cations would result in the observed hyperkalemia and acidosis.

Anything which would increase the delivery of NaCl to the distal segment should correct these abnormalities. Consequently Dr. Kennedy administered Na_2SO_4, and the resultant osmotic diuresis increased the excretion of Na+, K+, titratable acidity, NH_3, and water. Hyperkalemia, acidosis, and hyponatremia improved.

A third disturbance which may interfere with urinary dilution is a reduced capacity to reabsorb sodium and thereby to free water in the distal segment. This may be a significant factor in adrenal insufficiency, as well as in those cases of chronic nephritis

associated with increased rates of salt excretion. In both, the sodium reabsorptive capacity of the distal segment is decreased despite adequate flow to this area, and consequently solute-free water may not be formed in large amounts. Both of these clinical situations may be complicated by an associated fall in ECF volume and GFR as well as by increased secretion of ADH.

The final disturbance, "inappropriate" ADH secretion has been commented on already. The only thing I should like to add is that in view of the fact that there are so many non-osmotic factors which stimulate ADH secretion, such as pain, anesthesia, surgery, etc., that enhanced secretion of ADH may well be a ubiquitous factor in the pathogenesis of hyponatremia. Certainly post-operative hyponatremia may well be due to the combined effects of excess administration of intravenous fluids and persistent ADH secretion. Recently Drs. Schwartz and Bartter demonstrated that so-called pulmonary salt-wasting may well be due to the continuing secretion of ADH in the face of a low plasma osmolality. However, the early suggestion that this syndrome is due to a resetting of the osmostat as a result of inactivation or loss of cell cation, though not necessarily, may be consistent with their arguments.

I would like to conclude by stating again that any of a number of factors may contribute to the development of dilution hyponatremia. Furthermore, all hyponatremic patients do not have the same defects. This is borne out by the fact that there is an obvious difference in the pathogenesis of the hyponatremia in the edematous nephrotics studied by Dr. Walser and myself and Dr. Kennedy's cardiacs. In both, Na+ reabsorption was sufficient to produce edema, and yet only in the latter group did it contribute to the development of hyponatremia. Some of you will note that I have not discussed the so-called inactivation or loss of cell cation in any detail. This, too, may be a factor in some patients with hyponatremia although most of the available evidence does not lend strong support to this view.

Thank you.

CHAIRMAN METCOFF: Are there any comments or questions?

DR. HOLLIDAY: Dr. Orloff, do you know if the people with pulmonary salt-wasting were able to tolerate a low salt intake, and reduce urine sodium output?

DR. ORLOFF. The patients whom Drs. Schwartz and Bartter studied were not able to tolerate a low-salt intake, as far as I can recall. They continued to excrete sodium when placed on a low salt diet.

The patients with pulmonary disease whom we studied were able to decrease their salt excretion after some time but not to the level one would expect were they normal. None developed signs of acute salt depletion. Most of the ones we studied had pulmonary tuberculosis, the ones they studied had neoplastic disease.

CHAIRMAN METCOFF: What is the role of cellular breakdown with release of water and solute into the extracellular phase in the presence of acute renal insufficiency? Does this contribute to hyponatremia and acidosis which may develop without excess fluid intake, as body weight is decreasing?

DR. ORLOFF: I am not at all clear how it can contribute to hyponatremia, unless you are talking about resetting of the osmostat, or inactivation of cell cation.

CHAIRMAN METCOFF: The observations suggest an increment of extracellular water to which has been added cellular water which is low in sodium.

DR. ORLOFF: Yes, but under those circumstances, were the pituitary acting normally, you would expect that the water would be excreted.

CHAIRMAN METCOFF: Unless one has acute renal insufficiency with marked oliguria or anuria.

DR. ORLOFF: Yes.

DR. HOLLIDAY: What happens to the patient with cardiac failure, whom you described? Will alcohol, under these circumstances, presumably block antidiuretic hormone secretion?

DR. ORLOFF: Dr. Kennedy did not administer alcohol, as far as I know, although others have administered it with success.

DR. THOMAS KENNEDY (National Institutes of Health). Our thesis was that this syndrome was not induced by ADH but that it could be interpreted on the basis of a low sodium chloride load to the distal segment.

DR. HOLLIDAY: Is it your feeling that the extraction of water in the final segment, i.e., collecting tubule, is influenced by antidiuretic hormone?

DR. ORLOFF: Yes; I think it is. Drs. Berliner and Davidson have shown that the removal of water in this segment does not require the presence of ADH; but it has also been shown that the urine may be concentrated further when ADH is administered. So I think it is perfectly reasonable to assume that the membrane even in the collecting system is responsive to ADH, and that more water diffuses out when ADH is present in both the diluting segment and the collecting ducts.

DR. HOLLIDAY: As to the relative effect of regulating diffusion with antidiuretic hormone the patient with cardiac disease might demonstrate that with alcohol administration.

DR. ORLOFF: By what route?

DR. HOLLIDAY. If some component of cardiac failure were responsible for antidiuretic hormone secretion, blocking it with alcohol would decrease water reabsorption and diminish the tendency to hyponatremia.

DR. ORLOFF: Actually both cirrhotics and cardiacs, as you know, have responded to alcohol with resultant water diuresis. A lack of response does not argue against the thesis of persistent ADH secretion or resetting of the osmostat, however. Parenthetically, I would like to quote Dr. John Peters, who once said he thought the administration of alcohol by vein was wasteful and an unesthetic way to administer alcohol to anyone. (Laughter)

DR. HOLLIDAY: In m defense, I did not specify a route.

DR. SCHREINER: Where do you classify the salt-losing syndrome following release of urinary tract obstruction?

DR. ORLOFF: I have never seen such a patient, although I have read a number of reports concerning them. Actually, I have found it difficult to ferret out the basis for the polyuria and salt loss.

The patients reported had invariably received an enormous amount of fluids prior to their admission to the hospital so that one might expect polyuria or the delivery of the excess fluid on the relief of obstruction.

DR. BARNETT: Do I understand you to say you have observed increased rates of urine flow after administering alcohol to patients with nephrosis?

DR. ORLOFF: I do not think we ever administered alcohol to a nephrotic. We have noted diuresis following water ingestion, however, which is a simpler procedure and may be interpreted in the same manner, that is, interference with ADH secretion.

DR. BARNETT: We have given alcohol by mouth to a few children with nephrosis and observed no increase in rate of urine flow.

DR. MARVIN LEVITT (Mt. Sinai Hospital, New York City): Were these patients dehydrated or in water diuresis?

DR. BARNETT: They were edematous.

DR. LEVITT: Were they making maximally concentrated urine?

DR. BARNETT: They were making highly concentrated urines; yes. They were excreting very low urine volumes of high specific gravity. They were not water-loaded. The reason I hesitate is that it is hard for me to think in terms of a dehydrated edematous nephrotic. These were neither loaded nor thirsting.

DR. CHARLES FOX, JR. (Flower-Fifth Avenue Hospital, New York City): In the patients described in the slides, Jack, can you tell us something about the composition of their urine? You discussed sodium and water, but your figures refer to osmolality, which means a lot of other substances. I do not know whether we can infer what is happening to sodium. Did these patients receive mannitol? What fraction of the urine solute was sodium?

DR. ORLOFF: A very small fraction of the excreted solute was sodium in the edematous patients.

DR. FOX: How can we discuss osmolar relationships here with respect to sodium when we do not know what is happening to sodium in the urine?

DR. ORLOFF: What I have been talking about is the excretion of water in excess of sodium. This is the only important thing with respect to correction of hyponatremia.

DR. FOX: Was there a change in the urine sodium level during this time?

DR. ORLOFF: There is a slight increase in sodium excretion, even in an edematous patient, when you give mannitol, but not an appreciable increase. However, the excretion of free water increases markedly and since this is essentially sodium-free, the concentration of Na+ in plasma rises. Even in the absence of ADH, the membrane in the diluting segment, we believe, is partially permeable to water, so that, if any solute remains within the tubule, it will restrain the outward diffusion of water and free water excretion will increase.

DR. FOX: I find it difficult to see that we are understanding more about the excretion of sodium.

DR. ORLOFF: I am not talking about the excretion of sodium. If we excrete water without sodium, we are going to increase the concentration of sodium in the plasma; and that is what one tries to do in the correction of hyponatremia.

DR. FOX: Actually, do you get out more water than sodium?

DR. ORLOFF. Certainly.

DR. FOX· You said the concentration hardly changed.

DR. ORLOFF: No. I said the rate of excretion increased just slightly. The excretion of sodium-free water increased markedly. So you get out relatively more water than sodium in these patients when you administer an osmotic diuretic. Otherwise, you could not change the plasma Na+ concentration.

DR. HOLLIDAY: Do you have any explanation for the gradually rising free-water clearance when you use mannitol as the diuretic, none of which is presumably reabsorbed?

DR. ORLOFF This is essentially what I was telling Dr. Fox. Our idea is that the membrane is partially permeable to water even when ADH is absent and that there is less outward diffusion of freed water when osmotically active solute is in the urine; that is, the solute itself restrains the outward diffusion of water.

DR. HOLLIDAY: Also, you may have more sodium reabsorbed in your second segment.

DR. ORLOFF· That may also be a factor.

DR. WILLOUGHBY LATHEM. The situation in the Addisonian patient, I assume, you would interpret, then, as being a lack of ability to reabsorb the sodium in that second phase; and that is the reason he cannot excrete the water load.

DR. ORLOFF· Partly. The capacity of the distal segment is undoubtedly decreased. Some investigators also think increased ADH secretion is a factor.

DR. BRADFORD: Do you think it is somewhat analogous, then, to the low sodium syndrome described here?

DR. ORLOFF: Yes; I think a low sodium in an Addisonian means the same thing as in anyone else. It means that water has been retained in excess of sodium. The first thing which may happen in the sequence of events in an Addisonian is excretion of extracellular fluid. This does not change the concentration of sodium in the plasma. Then other factors come into play, a fall in filtration rate, perhaps increased ADH excretion, etc. So they are unable to excrete ingested water; and they become diluted.

DR. BRADFORD: Possibly there is a common basis in both types of patients related to adrenal hormones. I hate to bring the word up.

DR. ORLOFF: I don't think so. There is one thing which is certain. The Addisonian is not secreting salt-retaining adrenal hormone, whereas the cardiac presumably is, and at an increased rate. So I think, in that sense, they are entirely different.

DR. LATHEM: Why do the hyponatremics continue to drink water?

DR. ORLOFF: I do not know what the answer is. There is one possibility; (and, I think, a reasonable one) and that is that drinking is a habit. Therefore despite dilution of body fluids the hyponatremic continues to drink in response to habit. If one restricts water, they obviously will not get hyponatremia.

DR. FOX: Do you know that?

DR. ORLOFF: Yes. If you restrict water long enough, they are certainly not going to remain hyponatremic. If one restricts water intake, it is difficult to cure hyponatremia, but it can be done. I unfortunately did not bring a slide showing that this is essentially what we did do in four of these patients. Obviously, insensible loss of water will be sufficient to reconcentrate the sodium.

DR. FOX: Are they comfortable under those circumstances?

DR. ORLOFF: No.

DR. FOX: I think you must distinguish between an excessive intake of water and excessive dehydration, on the other hand. Nevertheless, one still has hyponatremia as an important fact. The hyponatremia of the nephrotic syndrome is not simply from drinking too much water.

DR. ORLOFF: What else is it due to?

DR. FOX: I do not know; but I do not think the answer is unequivocably that.

DR. ORLOFF: I think there is no possible way of lowering the sodium, the osmotic pressure, unless more water is retained relative to solute in the body.

DR. FOX: There is another possibility which you dismissed; that is the removal of sodium into the cell.

DR. ORLOFF: How will that produce hyponatremia?

DR. FOX: Then the level of extracellular sodium will be reduced.

DR. ORLOFF: But water will move into the cells, since the osmotic pressure of the cells must rise if electrolyte enters. If sodium moves into cells in exchange for potassium on the other hand, the osmotic pressure will not change, nor will there be an appreciable change in plasma Na.

DR. FOX: Isn't that hypothetical?

DR. ORLOFF: I don't think so.

DR. DAVID S. BALDWIN (New York, New York): I would like to ask a question about dividing free water clearance by filtration rate in these patients. It is true that free water clearance per 100 cc. glomerular filtrate may be normal in that sense; but wouldn't those individuals with reduced filtration rates have a diminished total free water clearance?

DR. ORLOFF: Oh yes, I agree entirely. But, in the patients with the 13 cc. clearance, the free-water clearance under these circumstances actually was something like 2-3 cc. a minute, so that the daily free-water excretion approximated 3 L.

DR. LATHEM: But suppose you plotted, Jack, free-water clearance per free-water intake. Wouldn't it be very much reduced?

DR. ORLOFF: Oh, certainly.

DR. LATHEM: We got the impression it could not account for the hyponatremia.

DR. ORLOFF: The reduced excretion alone, if associated with suppression of ADH secretion could not account for the development of hyponatremia unless 3-4 L. of water/day were administered. This does not mean that a reduction in filtration rate is not an important contributory factor.

CHAIRMAN METCOFF: I think we could go on picking on Jack Orloff all the rest of the afternoon; but we must proceed with the program.

The next paper will be by Dr. Davis. Jim!

B. Aldosterone and Sodium Retention in Dogs with Experimental Ascites

DR. JAMES O. DAVIS (National Heart Institute). The data I will present this afternoon are concerned with the mechanism of sodium retention in dogs with experimental ascites.

In regard to electrolyte metabolism, these animals with ascites are very similar, in many respects, to patients with clinical edema, that is, to patients with cardiac failure, cirrhosis of the liver, or to patients with a nephrotic syndrome. They do not,

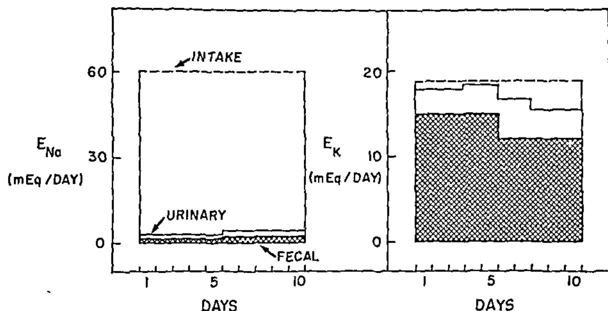


Fig. 63. Sodium (Na) and potassium (K) excretion (E) in mEq/day in urine (open area) and feces (cross-hatched area) in a dog with thoracic caval constriction and ascites.

Fig. 64. A natriuresis resulted following adrenalectomy and discontinuation of DCA therapy in a dog with thoracic caval constriction and ascites. See Fig. 63 for description of symbols.

of course, have a primary renal lesion. It has been our purpose to evaluate some of the possible mechanisms leading to sodium retention in these animals and, in particular, to study the relation of the adrenal cortex to the decreased renal excretion of water and electrolytes.

Before presenting these data, however, I would like to acknowledge that this series of studies represents the cooperative efforts of a number of physicians whose names have appeared on our definitive publications and who have contributed to the development of concepts and in carrying out these studies.

We have studied the relation of aldosterone to salt and water metabolism in a number of chronic animal preparations with ascites, but we have found two preparations most efficacious for these studies. These preparations are 1) dogs with thoracic inferior vena cava constriction and ascites and 2) dogs with right heart failure produced by controlled progressive pulmonic stenosis. Most of the data I plan to present this afternoon were obtained from study of the first preparation, the dog with vena cava constriction. I will now describe this preparation very briefly.

A heavy silk ligature is used to constrict the thoracic inferior vena cava. The constriction results in elevated venous pressure below the ligature and ascites forms. These animals accumulate large quantities of ascitic fluid and live for many months. Cardiovascular function and electrolyte excretion are sufficiently stable for chronic observations to be made before and after a given intervention. The animals tolerate other experimental surgical procedures very well such as bilateral adrenalectomy and total hypophysectomy.

DR. FOX. How big is the remaining opening in the vena cava?

DR. DAVIS: The diameter of the thoracic inferior vena cava is reduced to one-third to one-half of the original diameter in most dogs. Consequently, the cross-sectional area is reduced more.

Next, I would like to describe the pattern of electrolyte excretion in sodium retention. Figure 63 shows sodium and potassium excretion in a typical dog with thoracic caval constriction and ascites. On an intake of 60 mEq. per day, sodium retention was virtually complete, the animal was excreting only 1-2 mEq. per day of sodium in the urine and only 1-2 in the feces. In contrast, fecal potassium output was markedly increased, to a level of 12-15 mEq. a day, compared with a normal excretion of 1-2 mEq./day. Urinary potassium output was reduced.

Very early in this series of studies, we evaluated the importance of a low cardiac output and a low glomerular filtration rate in the retention of sodium, but found no evidence to suggest that either of these factors is of primary importance.

The first indication that the adrenal cortex is important came from studies of fecal electrolyte excretion. The low sodium and high potassium excretion in the feces (Fig. 63) is the pattern which has been reported by others for sweat and saliva in the presence of large amounts of a sodium-retaining hormone such as DOCA. This suggested that there might be increased amounts of sodium-retaining hormones in dogs with ascites.

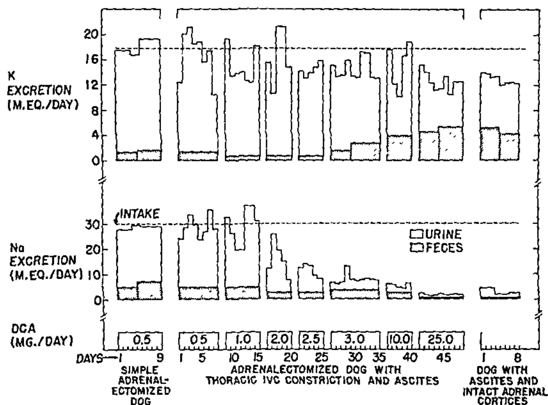


Fig. 65. The effects of different doses of DCA on sodium and potassium excretion in an adrenalectomized dog with thoracic caval constriction and ascites. For comparison, the efficacy of 0.5 mg./day of DCA for maintenance of the simple adrenalectomized dog is shown on the left and the pattern of electrolyte excretion in the dog with caval constriction and intact adrenals on the right.

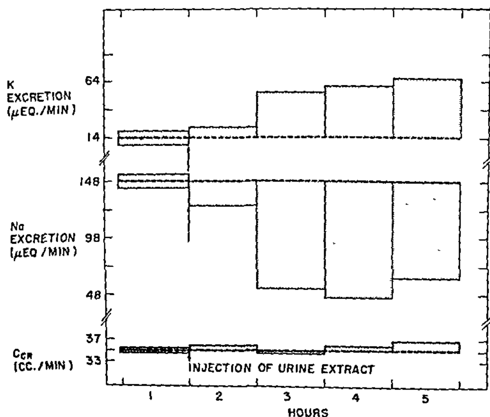


Fig. 66. The effects of intravenous injection of an extract of urine from a dog with experimental ascites on urinary Na and K excretion and renal creatinine clearance in an adrenalectomized assay dog.

Consequently, these data led us to study the effect of adrenalectomy upon electrolyte excretion in dogs with ascites secondary to caval constriction. If an excess of sodium-retaining hormone is present in these animals, and you take out the adrenal cortex and take them off hormone therapy, you should get a sodium diuresis. Data to illustrate the sodium diuresis are presented in Figure 64. In a dog with caval constriction, the right and left adrenals were removed and therapy was discontinued. A striking increase in sodium excretion occurred and sodium balance became negative. After all ascites disappeared, signs of acute adrenal insufficiency supervened.

We reasoned, then, that in an adrenalectomized animal with caval constriction one should be able to reproduce the pattern of electrolyte excretion shown in Figure 63 in every detail by administration of sufficient DOCA. The results from such a study are presented in Figure 65. In the center section is presented the effects of administration of doses from 0.5 to 25 mg./day to an adrenalectomized dog with thoracic caval constriction and ascites. For comparative purposes, on the left is shown the effect of 0.5 mg./day of DOCA in a simple adrenalectomized dog, and on the right is shown electrolyte excretion in a dog with thoracic caval constriction, ascites and intact adrenals. To return to the center section, the adrenalectomized dog with caval constriction on 0.5 mg./day of DOCA is in sodium balance. This animal had about two liters of ascites at this time and ascitic fluid was neither being formed nor lost. This situation of sodium balance is the same as observed in normal dogs and, of course, the same as that found for the simple adrenalectomized dog on adequate DOCA. When the dose of DOCA was progressively increased, a drop in both urinary and fecal sodium excretion occurred until, with a large dose of DOCA of 10-25 mg./day, sodium excretion in both urine and feces was the same as in a dog with caval constriction and intact adrenals.

Looking next at potassium output, you will notice there was a progressive increase in fecal potassium, a progressive drop in urinary potassium until, with large doses of DOCA, we observed the same pattern as in the dog with intact adrenals and ascites shown on the right. We were able, therefore, to replace the pattern of electrolyte excretion shown on the right rather completely when we gave large amounts of DOCA to the adrenalectomized dog with caval constriction.

We have carried out similar studies on nine adrenalectomized dogs with heart failure, and found, again, that the degree of sodium retention was related directly to the amount of sodium-retaining hormone (in this case, DOCA) given.

This indirect evidence from studies of adrenalectomized dogs led us to study aldosterone, the sodium-retaining hormone of the adrenal cortex. Our first observations were made on urinary aldosterone output. Urine was extracted with methylene chloride and the extracts were injected into bilaterally adrenalectomized dogs maintained on a high-sodium diet, and assayed according to the method developed by Dr. Grant Liddle [1].

[1] Liddle, G. W., Cornfield, J., Casper, A. G. T. and Bartter, F. C. The physiological basis for a method of assaying aldosterone in extracts of human urine. J. Clin. Invest. 34: 1410, 1955.

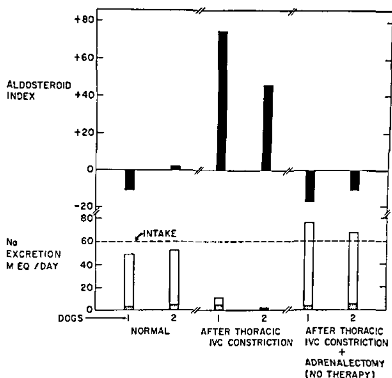


Fig 67. Effects of thoracic caval constriction and subsequent bilateral adrenalectomy upon aldosterone and sodium excretion in two dogs. Each value for urinary sodium excretion (open area) and for the aldosteroid index represents the average for two 4-day urine collections.

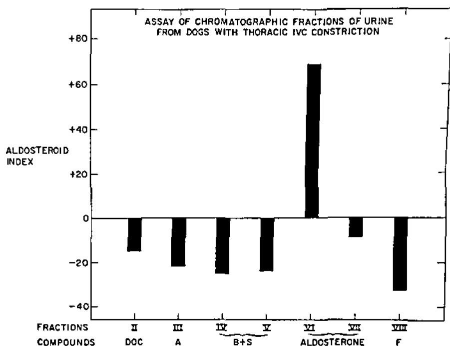


Fig. 68. Results from assay of fractions obtained by paper partition chromatography of two separate 8-day urine collections. Sodium-retaining and increased potassium excreting activity was found only in the aldosterone fraction.

A typical response to the *injection of an extract of urine from* a dog with thoracic caval constriction and ascites into a bilaterally adrenalectomized assay dog is presented in Figure 66.

After a one-hour control period, the extract was injected intravenously. A striking drop in sodium excretion and an increase in potassium output occurred; glomerular filtration rate, as measured by creatinine clearance, was unaltered. These changes indicate a direct effect of aldosterone on the renal tubular transport of sodium and potassium. Extracts of urine collected from normal dogs for a similar period of time showed no response.

The following slide (Fig. 67) shows the effects of thoracic caval constriction and subsequent adrenalectomy upon urinary aldosterone and sodium excretion in two dogs. The aldosteroid index *is a quantitative expression of the amount of aldosterone*; it represents the additive effects of a drop in sodium excretion and an increase of potassium output. The experiment was begun with two normal animals on an intake of 60 mEq. per day of sodium. Aldosterone was undetectable in urine and the animals were in sodium balance. Then, we constricted the thoracic inferior vena cava. Sodium retention became almost complete and large amounts of aldosterone were excreted in the urine. Finally, after adrenalectomy of the animals and discontinuation of hormone therapy, there was no aldosterone detectable, and a sodium diuresis occurred.

Evidence that the material which we have assayed was aldosterone was obtained by chromatographing the urine extracts and assaying the various fractions in bilaterally adrenalectomized dogs. The next slide (Fig. 68) shows the results from one such study. You will notice that sodium-retaining and increased potassium-excreting activity was present in only one fraction, the aldosterone fraction.

So, from the evidence presented, it seems fairly certain that increased circulating aldosterone is the hormone associated with the marked sodium retention in dogs with *experimental ascites*. This brings us, then, to the *important* question of the source of increased circulating aldosterone. Is it the result of increased secretion by the adrenal cortex; or it is the *result of decreased degradation* of aldosterone by some organ such as the liver, which we know is markedly congested in these animals? It is possible, of course, that both factors are involved.

We studied the possibility of increased secretion. The next slide (Fig. 69) shows our method of obtaining adrenal vein blood for study and for measurement of the rate of aldosterone secretion. Adrenal vein blood was obtained by cannulation of the right adrenolumbar vein. Then, by occlusion of this vein at its entrance into the inferior vena cava, blood was collected by retrograde flow into a graduated cylinder. Blood obtained in this manner was extracted for aldosterone, chromatographed, and the various chromatographic fractions assayed for sodium-retaining and potassium-excreting activity in the bilaterally adrenalectomized dog. The results showed activity in only one fraction, the aldosterone fraction.

The next slide (Fig. 70) shows the results from observations of the rate of aldosterone secretion, expressed on the ordinate as micrograms per hour, *in a group of* normal dogs and in a group of animals with experimental ascites, including both dogs

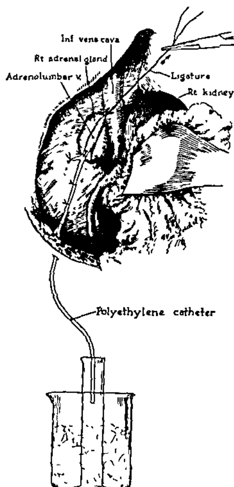


Fig. 69. Right adrenal gland and associated structures showing method of collection of adrenal vein blood.

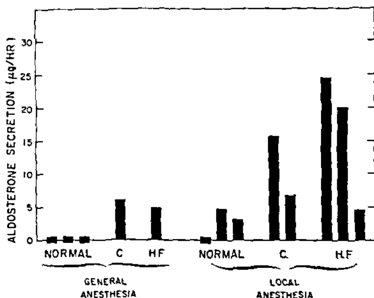


Fig. 70. The rates of aldosterone secretion in normal dogs, dogs with thoracic caval constriction (C) and dogs with right heart failure (H.F.)

with caval constriction and dogs with heart failure. The first five animals were studied under nembutal anesthesia. We were unable to detect aldosterone in 300 cc. of blood from normal dogs because the assay was not quite sensitive enough. However, we did detect it, in two experimental animals, in 300 cc. of blood. When the data were expressed in terms of secretion, the rates were five and six micrograms per hour.

In the studies of these first five dogs, we noticed that venous pressure dropped markedly in the two dogs with experimental ascites and we thought that the nembutal anesthesia might be partially turning off the aldosterone mechanism. Therefore, the remaining animals were studied under local anesthesia. Under this circumstance, aldosterone was detectable in normal dogs and the rates of secretion were measured in two of the three normal animals. In four of the five dogs with ascites, aldosterone secretion was considerably higher than in the normal dogs. When all the data, normal versus experimental, were treated statistically, a P value of approximately .01 was obtained which showed a higher secretion rate in the dogs with experimental ascites.

More recently, we have started another series of observations in which we have confirmed these findings. In these acute studies, we begin the experiment with a normal dog and make control observations on the rate of aldosterone secretion. Then, we constrict the thoracic inferior vena cava and follow aldosterone secretion for 4-5 hours. From this experiment, we have found that the rate of aldosterone secretion increased within 15 to 30 minutes after application of the ligature.

This brings us to the still more important question of what stimulates the adrenal cortex to secrete aldosterone. One of the first possibilities considered by us and, I am sure, by most people who studied this problem, was whether there is increased output of ACTH, with a subsequent increase in the rate of aldosterone production. We have found that the anterior pituitary gland is not essential for increased aldosterone output, sodium retention, and ascites formation. In fact, before methods for measuring aldosterone were available, we studied this problem in hypophysectomized dogs with caval constriction and ascites. When the venous pressure was high enough, we routinely observed almost complete sodium retention. This occurred, of course, in the presence of an atrophic adrenal cortex.

More recently, we have repeated these studies and measured aldosterone output in addition to sodium excretion. The results from observations on three animals are presented in Table 22. In this experiment we studied the effect of constriction of the thoracic inferior vena cava upon aldosterone and sodium excretion in three hypophysectomized dogs. We began the experiment with three normal dogs on each of which a total hypophysectomy was performed. Then, we set the animals aside for about six weeks to allow the adrenal cortex to atrophy. Following this time control measurements which are shown in the left part of the table were made. Aldosterone was undectable in four days of urine from the three simple hypophysectomized dogs and the animals were in sodium balance. Sodium intake was 60 mEq. a day. When the thoracic inferior vena cava was constricted, all three dogs formed ascites. Observe the increased output of aldosterone in dogs 8 and 9 and the drop in sodium excretion in the same two animals, from 54 to 9 and 51 to 5. In dog 10, ascites formed but was present for only one or two days. The assay for aldosterone from dog 10 was determined on a four-day urine and we were unable to detect an increased output of aldosterone possibly because of a transient elevation. Also, sodium excretion per day was higher in dog 10 than in dogs 8 and 9.

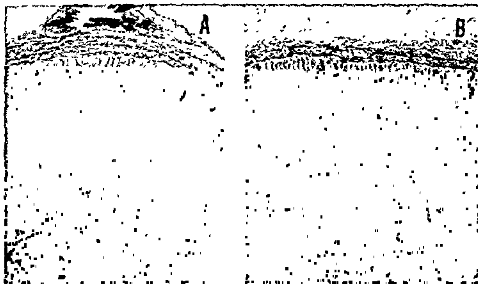


Fig. 71. The adrenal cortex from a normal dog (A) and from a hypophysectomized dog with thoracic caval constriction and ascites (B). In the adrenal cortex from the hypophysectomized dog, there is marked atrophy of the two inner layers while the zona glomerulosa appears unaltered.

TABLE 22

EFFECTS OF CONSTRICTION OF THE THORACIC INFERIOR VENA CAVA ON URINARY ALDOSTERONE AND SODIUM EXCRETION IN HYPOPHYSECTOMIZED DOGS

	Hypophysectomized		Hypophysectomized after IVC constriction	
Dog	Aldosterone μg/day	Na Excretion mEq/day	Aldosterone μg/day	Na Excretion mEq/day
8	<0.9	54	8.0	9
9	<0.9	51	1.7	5
10	<0.9	45	<0.9	14

The histology of the atrophic adrenal cortex of dog 9 is shown in Figure 71b; for comparison, the adrenal cortex from a normal dog is presented in Figure 71a. You will recall that dog 9 was excreting an increased quantity of aldosterone in urine (Table 22). The zona glomerulosa of the adrenal cortex of dog 9 appeared normal but the zona reticularis and zona fasciculata were markedly atrophied.

These data suggest 1) increased output of aldosterone from the zona glomerulosa although the observations do not exclude some secretion of aldosterone from the two atrophied layers, 2) that the anterior pituitary gland is not essential for increased aldosterone secretion, sodium retention and ascites formation, and, finally, 3) that the role of the anterior hypophysis is not a primary one in the aldosterone mechanism.

Similarly, it has been shown by other workers[2, 3] that the neurohypophysis is not essential for chronic sodium retention and ascites formation.

The one factor which we have consistently found necessary for this mechanism to go into action is a high venous pressure above the hepatic outflow into the inferior vena cava. This is not a direct effect of a high venous pressure upon the adrenals or kidneys because, if a caval constriction is placed above the renal and adrenal veins, chronic sodium retention and ascites do not occur.

We feel that the most plausible hypothesis for the sequence of events leading to sodium retention is the one Dr. John Peters proposed several years ago; namely, that *there is a high venous pressure*, with a consequent loss of fluid and electrolytes from the blood stream, and that some factors occurring in association with this fluid and electrolyte loss lead to stimulation of the adrenal cortex, increased aldosterone secretion, and, finally, sodium retention.

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[2] Hamilton, W. F., Ellison, R. G., Pickering, R. W., Hague, E. E., and Rucker, J. T., Hemodynamic and endocrine responses to experimental mitral stenosis. Am. J. Physiol. 176: 445, 1954.

[3] Laragh, J. H., Van Dyke, H. B., Jacobson, J., Adamsons, K. Jr., and Engel, S. L., The experimental production of ascites in the dog with diabetes insipidus. J. Clin Invest. 35 897, 1956.

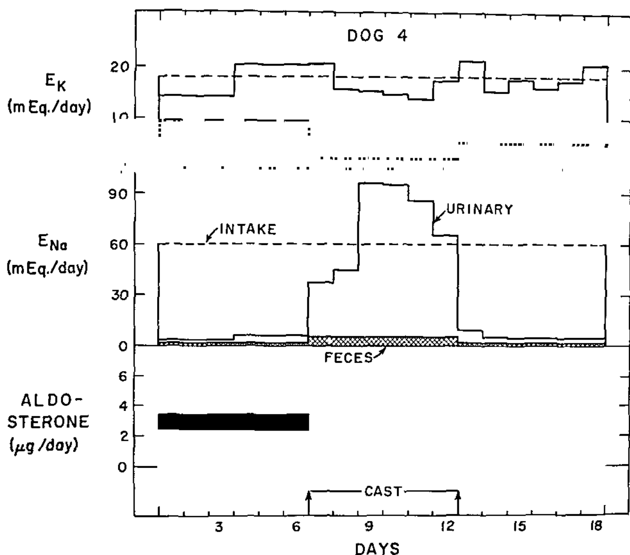


Fig. 72. Effects of a body cast on aldosterone, sodium and potassium excretion in a dog with thoracic inferior vena cava constriction and ascites. *See Fig.* 63 for description of symbols and legend.

We reasoned that, if this hypothesis is correct, we should be able to turn off the aldosterone mechanism by inhibiting or preventing the loss of fluid and electrolytes from the blood stream. To accomplish this, we applied a plaster body cast to dogs with caval constriction, and studied the effects upon aldosterone output and sodium excretion.

The results from the study of an animal with thoracic caval constriction to which a body cast was applied are presented in Figure 72. The experimental design employed is shown at the bottom of the slide, a control period, the period of the cast, and a recovery period.

During the control period, the animal showed the pattern of electrolyte excretion which I have described previously. There was almost complete sodium retention, a low rate of fecal sodium and a high fecal potassium excretion, and increased aldosterone output in urine. After the body cast was applied, there was a natriuresis and a return toward normal of the fecal electrolyte excretion. This change in fecal electrolyte output was indicative of a decrease in circulating aldosterone. More direct evidence of the decreased level of circulating aldosterone was obtained from the decline in urinary aldosterone. During the recovery period, these functions reverted to the control levels. We feel that the most likely explanation for the decreased aldosterone output following application of a body cast was an increase in intra-abdominal pressure which inhibited the filtration of fluid and electrolytes into the peritoneal cavity.

In summary, then, I would like to make three principal points:

First, that there is increased secretion of aldosterone by the adrenal cortex in dogs with experimental ascites,

Secondly, that the degree of sodium retention by the kidney is related to the amount of sodium-retaining hormone present; and

Finally, in closing, we would like to suggest that the most plausible working hypothesis for ascites formation in these animals is increased venous pressure, with a consequent loss of fluid and electrolytes from the blood stream, associated adrenocortical stimulation with increased output of aldosterone and, lastly, sodium retention.

CHAIRMAN METCOFF. This very interesting study is open for discussion.

DR. BALLY: Dr. Pollock in Dr. Kark's group in Chicago has reported a nephrotic syndrome occurring in patients with increased caval pressure (e.g., after thrombosis or with tricuspid insufficiency). (Lecture, PBBH, Spring 1957). I wonder whether such a syndrome was observed in these dogs?

DR. DAVIS. No. There was nothing to suggest a nephrotic syndrome. There was no albumin in the urine and the only pathological changes in the kidney were those secondary to chronic passive congestion.

DR. ROBERT E. COCKE (Johns Hopkins University and Hospital): What were the potassium concentration and plasma volume changes in the animals during the constriction phase?

DR. DAVIS: Plasma potassium was slightly elevated in most of the animals. By slightly elevated, I mean, from around 4 to 5-5.5 mEq/L. Plasma volume in the dogs with caval constriction was unchanged. In the dogs with heart failure, it was increased about 50 per cent as measured by T-1824 dye space.

DR. FOX: Have you any information on the amount of continual loss of potassium from these animals?

DR. DAVIS: There was not very much. Urinary potassium was reduced and, frequently, the animals were in positive potassium balance. If they were stressed markedly by the circulatory stress, some of the dogs with heart failure showed a negative potassium balance.

CHAIRMAN METCOFF: Dr. Davis, did you say that when you put a body cast on these dogs, you believed you were increasing the intra-abdominal pressure and, thereby, *producing a natriuresis?*

DR. DAVIS: This, of course, would decrease the effective filtration pressure which we are not able to measure, which is the factor that turns off the aldosterone mechanism.

CHAIRMAN METCOFF: This is quite interesting, because it seems to be precisely the opposite from the point of view Bradley and others postulated several years ago.

DR. DAVIS: The increases in abdominal pressure produced by the cast were not very high. There was no change in the hemodynamic function in the kidney.

CHAIRMAN METCOFF: After Bradley's report some years ago on several occasions we did paracenteses on nephrotic children with simultaneous renal functions. We wondered whether the paracentesis would result in a change in sodium excretion as a result of decreased intra-abdominal pressure. It did not. Nonetheless, nephrotic children with massive ascites occasionally diurese after paracentesis.

DR. DAVIS: We did the same measurements in dogs with experimental pericarditis and, we too, found no changes.

DR. LATHEM: Apparently, the position of the experimental subject may alter the sort of effect you would get from removing the fluid. Dr. Davis, what position were these animals in when you got the excretion?

DR. DAVIS: They were standing or lying in a natural position.

DR. LATHEM: They were standing upright.

DR. DAVIS: They were always in metabolic cages.

DR. LATHEM: These hourly excretion rates were not with the animals flat on their backs.

DR. DAVIS: The balance studies were done in metabolic cages. The assays on the adrenalectomized dogs were done with the dogs lying in a natural position and not on their backs. You cannot restrain the adrenalectomized dog very well without supportive hormone therapy without the blood pressure dropping very markedly. We just allow them to recline on a flat-top table.

DR. LATHEM: The studies in the casts were balance studies.

DR. DAVIS: That is correct.

CHAIRMAN METCOFF: Have you ever tried, Dr. Davis, putting a plastic casing around the liver, for example, near the constriction in the inferior vena cava? This might effectively do locally what you do with the body cast generally - i.e.: prevent the exudation and increase the intravascular pressure?

DR. DAVIS: I think one thing which may be confusing is that most of the ascitic fluid comes from the hepatic capillaries and lymphatics. That experiment, technically, would be rather difficult to do. Certainly, it is a very interesting thought.

DR. GRIBETZ: If you observe a child with "a little ascites" and study him as his ascites increases, might you not reach a point where it would "trigger off" a diuresis?

DR. DAVIS: Precisely. I think that is what occurs in these animals. In fact, we followed two dogs with caval constriction and ascites until the abdomen was markedly distended. The dogs came into sodium balance but remained in balance for only a couple of days and then they began to retain sodium again. Actually, the equilibrium is so labile, we found it necessary to put them into a cast in order to insure a diuresis.

DR. GRIBETZ: Then, exactly the opposite may occur. Frequently we perform a paracentesis, and shortly thereafter, a diuresis results.

DR. DAVIS: That must mean that there is another factor involved. (Laughter, applause)

DR. BARNETT: Did you make any assays of antidiuretic hormone in these observations?

DR. DAVIS: No, we did not. The only thing we have observed in this connection is that the hypothalamus of these animals contains a large amount of neurosecretory substance. This neurosecretory substance is a stainable material which seems to reflect the presence of ADH. We are currently trying to correlate the amount of neurosecretory material with the plasma sodium concentration. We have done no assays for ADH.

DR. BARNETT: There is a lot of skepticism about the methods used to assay ADH which I share. However, in almost every type of patient with edema in whom there has been demonstrated to be an increased rate of secretion of aldosterone there has been also an increase in antidiuretic hormone.

DR. BARNETT: And the dog develops ascites, which he did not have before.

DR. DAVIS: That is right.

DR. BARNETT: Then can that ascites be maintained if you continue giving the dog DOCA?

DR. DAVIS: Yes; they maintain it for weeks, months, as a matter of fact.

DR. LEVITT: This partly answers my question, it seems to me, that here is a circumstance under which one can maintain edema associated with DOCA over a period of time.

The only other subject I know who remains sensitive is the adrenalectomized or Addisonian subject; the normal individual does not.

DR. ROBERT BERLINER (National Heart Institute): I do not think it is quite true that you lose the salt-retaining effect of either the DOCA or aldosterone after the first week or so. There is no longer accumulation of edema; but the potassium loss, for instance, continues indefinitely; this is the evidence that the renal effect is still there.

I think the point is, as Jim said, that you need another factor; you have to have some place to put this extracellular fluid.

CHAIRMAN METCOFF: What happens with the potassium load in these animals?

DR. DAVIS: We have not studied this thoroughly. We did observe the effect of a potassium load in one animal with experimental ascites. Aldosterone output in the urine was increased.

DR. HOLLIDAY: Have you determined any factors other than increase in venous pressure, which will bring about the continuing retention of sodium?

DR. DAVIS: No; we have not. These are the preparations we have used most extensively; and the other preparations we have studied show essentially the same thing. The dog with auriculo-ventricular block, the dog with arterio-venous fistula, and the dog with pericarditis, all show essentially the same mechanism.

DR. BERLINER: Stahl actually has done that by plasmapheresing dogs, putting them on a low-protein diet, giving them DOCA, and putting a constriction on the portal vein. He has found it necessary to reduce the plasma protein concentration and put a constriction on the vein in order to get ascites. You do not get anything with just DOCA; but, if the animals have a place to put excess fluid, they will continue to retain fluid indefinitely.

DR. HOLLIDAY: In other words, that would be another circumstance.

DR. FOX: Is it possible to produce anasarca as well?

DR. DAVIS: Occasionally a dog with heart failure gets massive edema and anasarca. We have never been able to determine why they get so edematous. Most of the animals, however, do not get anasarca.

DR. FOX: Is it related to the plasma protein level?

DR. DAVIS: No; I do not think it is.

CHAIRMAN METCOFF: I am afraid we will have to turn off the discussion, for we must proceed. Following the next paper, on chlorothiazide in nephrosis, by Dr. Schreiner, we will have a coffee break.

C. Chlorothiazide in Nephrosis

DR. GEORGE E. SCHREINER (George Washington university): Dr. Metcoff and Members of the Conference: Chlorothiazide (which is a benzothiadiazine derivative) is an organic sulfur compound of high biologic activity, which has aroused considerable interest because of its ability to block sodium and chloride reabsorption in the renal tubule. It is a moderate carbonic-anhydrase inhibitor in vitro but, in dog and in man, produces a urinary electrolyte pattern more reminiscent of mercurial diuretics. It is potent, orally effective, and has produced no specific toxicity other than electrolyte imbalances resulting from its main action.

Previous communications from this laboratory[4, 5] have commented on its effectiveness as a diuretic in the edema of cirrhosis and congestive failure; on its enhancement of osmotic diuresis in some patients with chronic renal failure, on its antihypertensive effect in a patient with acute glomerulonephritis and other types of renal disease. The diuretic effect of chlorothiazide appears to be additive to, rather than competitive with, the sodium bicarbonate diuresis of acetazoleamide and the sodium chloride diuresis of organic mercurials.

The present report will be confined to studies of the diuretic action of chlorothiazide in patients with the nephrotic syndrome. The drug has been found to be a valuable adjunct in the management of five situations which commonly occur in the natural history of the nephrotic patient. These are:

1. To control edema in the patient with mild nephrotic syndrome, for whom no steroid therapy is planned;

2. To control or reduce edema during the first two weeks of initial steroid therapy, when the salt retention of the steroid may be additive to the nephrotic process.

3. To potentiate steroid-withdrawal diuresis in the refractory patient.

[4] Schreiner, G. E. and Bloomer, H. A., Effect of chlorothiazide on the edema of cirrhosis, nephrosis, congestive heart failure and chronic renal insufficiency. New England J. of Med. 257: 1016, 1957.

[5] Schreiner, G. E., Chlorothiazide in Renal Disease. N.Y. Academy of Sciences. To be published.

TABLE 23

CHLOROTHIAZIDE IN NEPHROSIS - ELECTROLYTE BALANCE STUDIES

Diagnosis	Renal Function	Sodium mEq/24 hrs.			Sodium Balance mEq	Weight Loss lbs.	Duration Treatment Days
		Dietary Intake	Control Excretion	Treatment Excretion			
1. Etiology unknown	BUN - 9	8	17	118	-329	-3	3
2. Lupus nephritis	BUN - 35	17	55	424	-2848	-46	7
3. Membranous G-nephritis	Ccr - 150	17	0	82	-131	- ½	2
4. Chronic G-nephritis	BUN - 30	8	17	106	-293	-6½	3
5. Membranous G-nephritis	BUN - 27	17	0	64	-94	-3½	2
6. Subacute G-nephritis	BUN - 35	8	4	13	-16	- ½	3
7. Renal amyloidosis	Ccr - 75	8	0	5	-	-2	3
8. Chronic G-nephritis	BUN - 105	17	45	76	-	-6	6
9. Membranous G-nephritis	Ccr - 102	8	71	191	-	-20	6

Source: Urinary sodium excretion in nephrotic patients treated with chlorothiazide, N.Y. Academy of Science - Diuretic Symposium.

4. To control weight during maintenance-steroid administration, and lessen the rigidity of salt restriction.

5. To enhance osmotic diuresis in the nephrotic patient who has progressed to moderate renal failure. Here sodium, potassium, and chloride are added to urea as the loading solute.

Table 23 summarizes the magnitude of the sodium excretion and weight loss in nine nephrotic patients (all tissue-proven except one) on whom electrolyte excretion studies were carried out. The renal function is indicated. The two levels of dietary intake were 8 and 17 mEq. of sodium. The patients were allowed to equilibrate on the diet for a few days before the control period. The mean urinary excretion for a two or three-day treatment is listed, and the duration of treatment is indicated in the last column.

The net sodium balance in milliequivalents during the duration of the particular treatment period may be compared with the weight loss in pounds in the adjoining column. As you can see, most of these patients achieved a marked negative sodium balance, except the patients with renal amyloidosis.

CHAIRMAN METCOFF: Most of those patients were in negative sodium balance to start with, I take it.

DR. SCHREINER: No, they were all edematous.

CHAIRMAN METCOFF: Let's take the first, second, fourth, and last two, all of whom had control sodium excretions exceeding their simultaneous sodium intake.

DR. SCHREINER: They were on a diet for three to five days, maximum; and this control period would be two days, so that they would be, at the most, seven days on a controlled dietary situation. Some of them were in slight negative balance but the order of magnitude is small compared with the treatment period. The third patient had no sodium in the urine.

The drug has been given to a total of seventeen nephrotic patients in nineteen courses; substantial diureses were obtained in twelve (Table 24). The range of weight loss is one-half to 46 pounds, the mean weight loss, 10.5 pounds and median weight loss in the series was 6 pounds.

TABLE 24

CHLOROTHIAZIDE IN NEPHROSIS - SUMMARY DATA

Total cases	17
Total courses	19
Substantial diureses	12
Range of weight loss - lbs.	0.5 to 46
Mean weight loss - lbs.	10.5
Median weight loss - lbs.	6

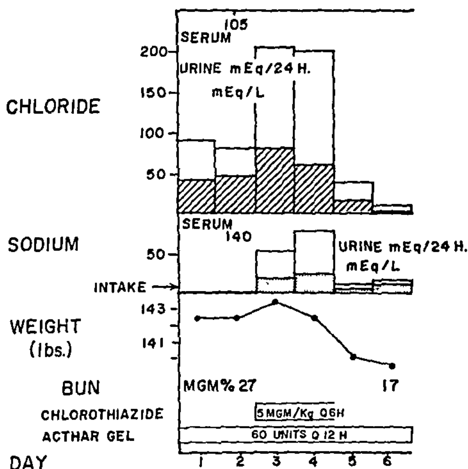


Fig. 73. Electrolyte excretion studies in a patient with membranous glomerulonephritis and nephrotic syndrome. Hatched area is the concentration in mEq/L. Total bar is excretion in mEq/24 hours. (New England Journal of Medicine)

Figure 73 illustrates a 59-year-old white female who had been using 1 per cent ammoniated mercury ointment for two years, and was admitted for edema which appeared three weeks prior to admission. Pitting, pretibial, and dependent edema were the only positive physical finding. Significant laboratory work included a rapid sedimentation rate (45 mm per hour), 4+ proteinuria, double refractile fat bodies, quantitative urine protein excretion of 7.6 grams, a total serum protein of 4.6 grams, with 1.3 albumin, a cholesterol of 604, a negative LE preparation and a renal biopsy which revealed diffuse membranous glomerulonephritis. The patient was placed on ACTH gel, 60 units, and received 5 mg. per kilo of chlorothiazide.

The intake was 8 mEq. per day; and you can see that there was no detectable sodium in the urine. The chloride concentration is indicated by the top of the cross-hatch, and the total excretion per day by the top of the bar.

On chlorothiazide, the patient had a sodium and chloride diuresis with a weight loss of 3½ pounds. After therapy was stopped the excretions returned to the control level.

After two weeks of steroid therapy, this patient went on to a full diuresis, with a 33-pound weight loss.

Figure 74 describes the results of an electrolyte study in a 71-year-old white male with the nephrotic syndrome and chronic glomerulonephritis proven by renal biopsy. Again, concomitant with the administration of chlorothiazide, there was a marked diuresis with weight loss. The patient was then placed on steroid. At this point he had no clinical edema. When chlorothiazide was reintroduced while on steroid, the patient did not have any increase in sodium excretion, but did have a slight increase in chloride excretion.

It has been our observation with other studies that, when the patient is on steroids in any form, the predominant cation may be potassium; and, upon cessation of steroids, it may change to sodium chloride. The chloride excretion may remain relatively constant.

Another example of chlorothiazide potentiating diuresis occurred in a 24-year-old white female with anasarca of four months' duration, purpura for ten days, and generalized bleeding. She had lupus for five years. She received many steroids for previous processes; but they had been stopped one month prior to admission. On physical examination, there was a blood pressure of 105/108, diastolic gallop, ascites, massive edema, parallel white lines on the fingernails. Laboratory work showed proteinuria of 6 to 8 g. in 24 hours, waxy, fatty, and granular casts, 10 to 15 red cells. Serum total protein was 3.4, with an albumin of 1. The platelet count was 1000.

She was started on 80 mg. of meticorten, to which chlorothiazide was added in a dose of 5 mg. per kilo. The patient had a very marked increase in sodium-chloride loss, with a weight reduction of fifty pounds over nine days.

Following this, the patient was given nitrogen mustard, and has subsequently had an apparently complete remission for a year.

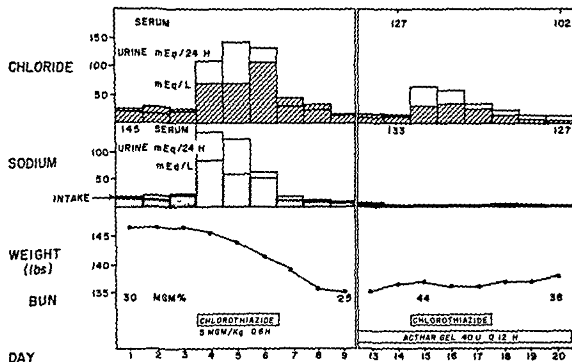


Fig. 74. Electrolyte excretion studies in nephrotic syndrome. (New England Journal of Medicine)

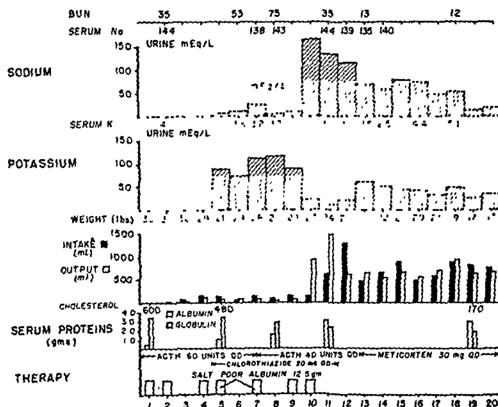


Fig. 75 Electrolyte excretion in an infant with nephrotic syndrome.

DR. BAXTER: How can you be sure the patient is not responding to steroids? How can you be sure the diuresis was due to chlorothiazide rather than to the increase in serum albumin?

DR. SCHREINER: In this particular case, we agree with you Jim, one cannot be certain. However, we have not seen diuresis due to steroids occur in the nephrotic syndrome without prior reduction in protein excretion. This patient's protein excretion did not fall off until quite some time later.

DR. BERLINER: Do the data you presented on this patient imply that there were 400 mEq. of some other cation excreted?

DR. SCHREINER: From the raw data, the discrepancies in concentration were 12, 24, 31, 76, 93, 107, and 112 mEq. respectively of chloride in excess of sodium on the first to seventh day of treatment in this patient.

DR. BERLINER: A patient cannot lose a hundred mEq. of potassium a day for very long.

DR. SCHREINER: Those are the data. (Laughter)

DR. FOX: Do you have any information on the serum sodium or chloride level?

DR. SCHREINER: The serum sodiums were 133, 138 and 134 mEq/L.

DR. FOX: And potassium?

DR. SCHREINER: The potassiums were 3.7, 4.2, 4.2 and 4.2 mEq/L during the treatment period.

This patient, incidentally, was comatose and areflexic when she came in. She was bleeding from every orifice, with ecchymoses right next to each other.

DR. COOKE: What happened to the serum chloride and CO_2?

DR. SCHREINER. I am afraid I do not have those at my fingertips, Bob. The patient clinically appeared to be acidotic in the beginning, because she was hyperventilating, but I do not recall what the CO_2 was. (Addendum - 20 mEq/L - the serum chloride of 124 mEq/L was reduced to 110 mEq/L.)

DR. ORLOFF: What is the significance of the parallel white lines on her nails?

DR. SCHREINER: This was described by Muehrcke in the British Medical Journal as being due to hypoalbuminemia; it has also been described in cirrhosis. We have been following this; and, in patients with acute malnutrition, it can develop in as short a time as ten days. So, it is not in the fingernail, it appears to be in the vascular bed. We think it has something to do with the distribution of edema near blood vessels. It is very common in nephrotics.

Figure 75 refers to a fifteen-month-old white female admitted for anasarca and oliguria of two weeks' duration. She was previously well, until six weeks previous to admission, when she had a throat infection, for which no therapy was given. Three weeks later, she developed hematuria, albuminuria, hypercholesterolemia, a positive urine, and throat culture for beta hemolytic strep. She was treated at that time with diuretics and meticorten, without any demonstrable improvement. Her weight increased from 22 to 30 pounds; and the anasarca was massive. Periorbital edema was so great as to produce mechanical blindness.

On administration of albumin, the blood pressure rose precipitously. Upon transfer to our hospital, the blood pressure was 170/100; the palpebral fissures were closed by edema. There was massive ascites.

Laboratory findings were albuminuria, the platelet count was 750,00, and rose to 1,100,000 on the second day, the sedimentation rate rising to 75. The patient was virtually anuric, with 3 cc. of urine representing the total collection for the first three hospital days. Serum albumin was zero; the globulin, 3.4; the cholesterol, 600. The patient had only been off steroids a day or two, and was continued on with ACTH, 60 units per day. Total urine collections were not feasible; so we did electrolyte concentrations in the urine, which was collected in a plastic funnel directly by the nurse.

The control urine is the first we got with 8 mEq. per liter of sodium, 88 of K. With intravenous chlorothiazide, the concentration of K rose to 118 mEq., as seen on the chart.

This patient had been getting albumin, which was continued; and the serum-albumin level rose, particularly when the frequency of albumin administration was increased.

We see that there was predominately a potassium effect from the chlorothiazide in this patient, until the serum protein became elevated, when there was an abrupt shift to sodium chloride excretion.

DR. LANGE: Couldn't that also be due to the ACTH administration?

DR. SCHREINER: Yes, probably. There was an abrupt diuresis.

DR. RILEY: Did you give any potassium by mouth?

DR. SCHREINER: No, we had to do it intravenously. This patient was too sick to take anything by mouth.

We are interpreting this as a nephrotic syndrome with acute glomerulonephritis with no response to steroids alone, chlorothiazide alone, or albumin alone. When prepared with steroids and elevation of her serum albumin by the addition of albumin and chlorothiazide, a prompt diuresis ensued.

In summary, chlorothiazide has been used in seventeen patients with a nephrotic syndrome. Electrolyte balance studies under controlled conditions were carried out in nine patients. They show a predominant chloruresis and natriuresis, except when the

patient is on adrenal steroids. Potassium may then be the dominant cation. Chlorothiazide was found to be a safe, potent diuretic, and a valuable adjunct in the treatment of the nephrotic syndrome.

With the Chairman's permission, I would like to make three capsule clinical observations to add to this. One is that, in a report which is to come out shortly in the American Journal of Medicine, we have reported a case of nephrotic syndrome in sickle-cell disease. The biopsies in this case showed miliary infarctions of the kidney, and thickening of the capillary basement membrane. We were unable to find any reference in the literature to the disease as a cause of the nephrotic syndrome. My purpose in bringing it up is to ask whether anyone in this group is aware of such reports. We have since seen two other cases; Dr. Meyers in Pittsburgh also has two cases. This may be a new cause of the nephrotic syndrome to look for. The histology is somewhat similar to what we have seen in patients with renal-vein thrombosis.

DR. PHILIP CALCAGNO (Buffalo, New York): There are reports of glomerulonephritis, certainly, with sickle-cell disease.

DR. SCHREINER: These patients did not have proliferative glomerulonephritic-type lesions; all they had was basement membrane thickening, but none of the other glomerular stigmata of glomerulonephritis.

The other two capsule observations I would like to make, in reference to the comments which were made yesterday, we had a patient who came into the hospital with granulomatous lesions in the lungs, following an acute respiratory infection with beta hemolytic streptococci. This was diagnosed by the X-ray department as Wegener's granulomatosis; and the patient was in acute pulmonary edema. We treated her with large doses of steroids. The pulmonary lesion reversed; and we biopsied her as soon as we were able to get her out of an oxygen tent. The biopsies showed proliferative glomerulonephritis; and the patient has had complete clearing of her clinical disease.

I bring this up because I wonder whether, in the experimental types of nephritis, we are not looking too exactly for one particular kind of histology. The report of one of the papers yesterday, showing granulomatous lesions which were produced in one of the experiments reminded me of this.

Another patient we have, with a very similar story, is a sixteen-year-old boy who was anuric for some sixty-odd days. He was treated with the artificial kidney. He started out with a classical, acute glomerulonephritis. In the third and fourth weeks, he began to develop lesions and bleeding. These were completely similar to the slides Dr. Germuth showed yesterday. He went on progressively to develop more widespread periarteritic lesions in other organs, while the kidneys showed proliferative, acute glomerulonephritis.

CHAIRMAN METCOFF: Thank you, Dr. Schreiner. Dr. Kennedy, would you care to comment about some of your studies on chlorothiazide?

DR. KENNEDY: My experience has been relatively limited. We have studied ten or twelve cases in congestive heart failure. The experimental protocols were

essentially the same. They were placed on a regimen of bed rest and low salt diet until weight and electrolyte excretion stabilized; then salt was introduced into their diet in increasing amounts, and at the same time, the drug was administered, to determine whether the addition of chlorothiazide would prevent accumulation of the edema in these people, or result in any further diuresis. A comment is in order on the type of cardiac with whom we dealt. More than half of our patients had essentially complete sodium retention, and any salt added to their diet was completely retained and reflected in weight gain. They were in just about as severe failure as is ever seen.

From our experience, we are not too sanguine about the effectiveness of chlorothiazide in this type of cardiac. An analysis of the series indicates that only about 50 per cent of the patients were significantly benefited by drug and these were the cases whose tendency to retain sodium was least striking. All of these patients were fairly resistant to diuretic agents; but weight loss could be induced and sodium excretion promoted in each after exhibition of mercurials with or without preloading with ammonium chloride and/or simultaneous administration of aminophylline.

We do not think the chlorothiazide is without effect on renal sodium transport. In those patients in whom there was no increment in sodium excretion after administration of the drug, a sharp increment in potassium excretion was observed and, in at least two or three cases, continuous exhibition of chlorothiazide was associated with the progressive development of relatively severe hypokalemic alkalosis. Our interpretation of this observation is that chlorothiazide reduces the transport of sodium in the proximal tubule thus increasing the load reaching the distal potassium-sodium exchange site. The increment in sodium reaching the exchange site is completely exchanged, and a roughly equivalent increment in potassium is excreted in the urine.

DR. HOLLIDAY: Do you believe that the drug inhibited carbonic anhydrase or had a mercury-like effect?

DR. KENNEDY: I would say there was no evidence that the drug showed carbonic anhydrase inhibitory activity. One further word of caution. Our experience with this drug may be atypical. We have heard by word of mouth, that other people are observing more favorable therapeutic results with chlorothiazide in the treatment of cardiac failure. The difference may be related to the severity of disease in the patients studied.

DR. McCRORY: We have had a very brief experience with two children, both of whom were refractory to any form of therapy, both nephrotics. They were given the drug in a lower dose; 10 mg. per kilo per day. Yours was 20 mg. per kilo per day. I do not know whether this is important; but, in a three-day and two-week trial, we saw no significant weight loss or diuresis in the two children.

Did you use any lower doses? Do you have any idea of the minimal effective dose?

DR. SCHREINER: No. Unfortunately, we took off from the dose which was reportedly effective in dogs, and tried higher dose levels, such as 15 mg. per kilo in patients. We demonstrated no increment of saluresis in patients by going to a higher dose. Apparently, it is an all-or-none situation, which levels off. But we have no studies below 5 mg. per kilo every six hours. I might say that, in patients with reduced filtration rates as you might expect, the success rate is much lower. In fact, you may get no results at all.

DR. McCRORY: These children were both in that category.

DR. GOODMAN: That is in line with what I was going to suggest. The patients he described were cardiacs, and perhaps more resistant to everything. We have treated one nephrotic with chlorothiazide who did not respond to it. Many of the patients in your first chart, I noticed, were putting out sodium (in fact, more than their intake), particularly the ones who apparently responsed to chlorothiazide. They were complex situations to evaluate, also being treated with steroid and albumin. I was wondering whether, in a series of refractory nephrotics on a low sodium intake and who were not putting out sodium, whether you would have any effect worth talking about.

DR. SCHREINER: I would not say they were all complex situations. In the first slide I showed you, the electrolyte excretion went up and then came down to the base line, directly in response to the drug. We have a number of patients who were treated without steroids.

In addition, I do not think that, if you start a patient on steroids, you can expect the steroid diuresis within a couple of days.

DR. GOODMAN: I say you are adding another factor. I would just be curious whether chlorothiazide alone would give a really good effect in many patients.

DR. SCHREINER: Nephrotics may be refractory for a variety of reasons. They may be refractory because they have a low filtration rate, they may be refractory if they have a very low concentration of serum albumin. In our experience people with less than 1½ g. per cent of albumin have difficulty getting a diuresis from any agent. This is just as true of chlorothiazide as of any diuretic agent I know of, so that, in patients with very low filtration rates and serum albumin, you may not get a diuresis.

CHAIRMAN METCOFF: I can hardly restrain myself at that, Dr. Schreiner. This would mean that virtually no children who have the nephrotic syndrome ought to diurese until their serum albumin is raised; but most of them do. Usually diuresis precedes elevation of serum albumin concentration.

DR. SCHREINER: You may not get a diuresis at the low level of serum albumin. You can raise the albumin and often get a diuresis in the same patient, simply by elevating the serum albumin.

DR. GOODMAN: In our series, the patients were all put on 200 mg. sodium diets. There was a diuresis in some which was very marked, diuresis in others was modest, and no diuresis occurred in others. There was no relationship between diuresis and their serum albumin level.

DR. SCHREINER: I am not making an absolute general statement; and I do not mean to imply that you can never get a diuresis in a patient with a low serum albumin. But I think everyone has had the experience of patients who are refractory to diuretic therapy, and in whom simple elevation of the serum albumin level is enough to initiate a diuresis.

CHAIRMAN METCOFF: Of course, infusion of concentrated human albumin does more than raise serum albumin concentration; it produces tremendous expansion of plasma volume. Volume expanders like Dextran also may induce diuresis.

DR. RILEY: I have had a very small experience also consisting of two patients, but in both these it was interesting that when we first started the chlorothiazide - this was without accompanying steroids - some diuresis began, but with this the serum potassium concentration went down to alarmingly low levels. Then we added extra potassium by mouth, and the diuresis slowed down. I was wondering if this was an experience anyone else had, or whether this was strictly coincidence.

DR. KENNEDY: What kind of diuresis? Water? Electrolyte?

DR. RILEY: Largely potassium diuresis.

DR. SCHREINER: Had the patients lost their edema by this time?

DR. RILEY: Oh, no. They had lost only a few pounds. Incidentally, they did drop their serum-sodium concentration pretty low, too; they seemed to put out salt without water which was a nice trick.

CHAIRMAN METCOFF: I think we have time for one more.

DR. ROBERT SCHWARTZ (Children's Medical Center, Boston, Massachusetts): Since everyone is reporting a small series, perhaps we ought to. We observed about six patients whom we classify in the difficult refractory group. The first patient, very briefly, was a girl who had two courses of ACTH without response, but with the development of hypertension. After her last course of ACTH, she got chlorothiazide in a large dose, ranging from 10 to 20 mg. per kilo, and had a complete diuresis, with sodium excretions well over 200 mEq. per day. She lost about 8 or 9 pounds without changing her protein excretion. She went on, with continuing nephrosis, but less edematous.

However, all the rest of the patients we treated did very poorly. One child, who was putting out around 1 or 2 mEq. of sodium per day, had nephrosis for a considerable period of time and did not respond in the slightest to large doses; however, subsequently, he was given ACTH and had an excellent diuresis.

Two or three other children increased their sodium excretion up to maybe 15 or 20 mEq./day; but they were on no added salt diets. This output was not large enough to cause a diuresis.

I might also mention a twelve-year-old girl, incidentally, who had had cirrhosis, with a previous splenorenal shunt and, more recently, had developed ascites and pleural effusion, who was excreting less than a milliequivalent of sodium a day in her urine, who, when given chlorothiazide, had marked potassium excretion, and became hypocholoremic without increasing her sodium excretion one bit.

DR. FOX: I know that time is short; but I do not think it amiss to point out that, after hearing about aldosterone and its effect, then hearing these different experiences about individuals having some good effect and some not good effect, I think we ought perhaps to wonder whether there are not several factors.

For example, if the sodium intake is extremely low, aldosterone activity could be maximal; and it would be hard to expect a drug like this to be as effective as when there is a reasonably significant sodium intake.

This is also true of steroids. I think we must not neglect the importance of the several factors operative here.

DR. SCHREINER: I think we should also comment on the fact that we know it works better with a better filtration rate; and in many of these patients reported here, filtration rates were markedly reduced.

In one patient of ours, the filtration rate was 75, and, when she was at bed rest, edematous, she had no demonstrable diuresis from the chlorothiazide. This patient had a very early amyloid infiltration and nephrotic syndrome. We decided to treat her with steroids. Her filtration rate has gone from 75 to 115; and she now gets a five- to six-pound diuresis with one day's administration of chlorothiazide. We have been able to keep her stable for six months.

DR. FOX: One of your most dramatic effects may have been misinterpreted. I would submit that you inhibit aldosterone by copious administration of the sodium salt without chloride; and I think this may have been significant.

DR. SCHREINER: I think this is a very good suggestion.

CHAIRMAN METCOFF: With that, I think we will recess for twenty minutes.

There will be a slight change in the program order. Dr Gribetz will present his material next. Dr. Gribetz!

D. Tissue Electrolytes in Nephrotic and DOCA Treated Rats

Dr. D. GRIBETZ (Mt. Sinai Hospital, New York). The studies which we are about to describe are still in progress. Although we have some hesitancy about reporting them because of this, we shall present the data accumulated to date.

The original purpose of these studies was to determine the tissue electrolyte composition of various forms of edema, utilizing the rat as the experimental animal. Experimental nephrosis in this animal was one of the forms of edema studied.

One of the objects was to determine whether stores of electrolytes in solid tissues such as bone and tendon, were mobilized during edema, as they appear to be in response to other stimuli such as acute acidosis.

During the course of the studies, it was shown that the solid tissues did not give up electrolytes in response to "nephrotic edema." When the tissue electrolyte composition was determined, however, it was decided to compare it to that found in the edema produced by DOCA and aldosterone administration. It became apparent that certain differences were present and these are now being presented with the hope that they may shed some light on the relationship of the salt-retaining hormones of the DOCA-aldosterone type to the edema of nephrosis or at least to that produced by experimental nephrosis in the rat.

Twenty-seven rats weighing between 50 and 130 gm. were made "nephrotic" by the Heymann technique. They were given three daily injections of anti-rat kidney rabbit serum and were sacrificed 1-3 days after the last injection.

Fourteen rats in the 300-350 gm. weight range were injected with 3 mg. of DOCA daily for seven days and were sacrificed one day after the final injection (the duration of the injections was chosen in order to correspond to the length of time necessary for the peak of edema to develop in the "nephrotic" animals).

Preliminary studies with aldosterone have just been started. Because of the difficulty in obtaining the drug, we have administered 30 micrograms per day for five days to only six rats thus far.

The data have been compared to two sets of control animals: twenty rats in the 50-150 gm. range and 20 rats weighing 300-350 gm.

All animals were maintained on the same diet, stock Purina chow, with water ad lib. All were killed by cardiac exsanguination under light ether anesthesia. Tissues analyzed were muscle, skin, tendon and bone.

Table 25 depicts the data obtained in the controls. The numbers outside the parentheses are the values obtained in the smaller animals used as the controls for the "nephrotic" rats; the numbers within the parentheses are the values obtained in the control group of the larger animals, used for comparison with the DOCA treated animals.

TABLE 25

NORMALS

	Na	Cl	K	H_2O
	mEq/g. dry tissue			gm/gm dry tissue
Muscle	.096 (.077)	.076 (.050)	.454 (.410)	3.32 (3.09)
Skin	.174 (.181)	.170 (.174)	.128 (.065)	2.14 (1.82)
Tendon	.239 (.188)	.209 (.191)	.071 (.020)	1.28 (1.25)
Bone	.273 (.287)	.036 (.030)	.028 (.020)	0.26 (0.23)
Plasma (mEq/L)	145 (142)	105 (106)	4.6 (4.0)	

They are all expressed as milliequivalents per gram of dry tissue except the water content which is expressed as grams of water per gram of dry tissue.

Several facts, which are well known, are obvious from these data (Table 25). 1) Muscle contains small amounts of sodium and chloride with large quantities of potassium. 2) Tendon has larger amounts of sodium and chloride and only meagre amounts of potassium. The quantities of sodium and chloride are greater than that which can be accounted for by calculating a fluid phase of the tendon. Therefore, it is assumed that a considerable quantity of these electrolytes is confined within the dense, solid phase of tendon. 3) Skin has larger amounts of sodium and chloride than does muscle, but much smaller quantities of potassium. 4) Bone has a large excess of sodium but contains only small quantities of chloride and potassium. 5) The importance of setting up two sets of controls is emphasized. The smaller animals have considerably larger amounts of sodium and chloride, at least in muscle. This we think is due to the larger extracellular volume possessed by younger animals.

Table 26 shows the data obtained in the "nephrotic" animals. First of all, it should be stated that when the animals were sacrificed, they demonstrated the plasma profile of the nephrotic syndrome. Their cholesterols averaged 373 mg. per cent in contrast to the control value of 82 mg. per cent; their mean total proteins were depressed to 3.4 gm. per cent in contrast to the control value of 5.5 gm. per cent. Their BUN's, however, averaged 56 mg. per cent in contrast to the normal mean of 24 mg. per cent (this elevation is apparently characteristic of "experimental nephrosis" in the rat in contrast to the "pure" human disease). Other plasma electrolytes were not significantly altered.

TABLE 26

"NEPHROTICS"

	Na	Cl	K	H_2O
	mEq/g dry tissue			gm/gm dry tissue
Muscle	.144 (.096)	.119 (.076)	.438 (.454)	3.78 (3.32)
Skin	.317 (.174)	.257 (.170)	.116 (.128)	3.20 (2.14)
Tendon	.240 (.239)	.227 (.209)	.057 (.071)	1.23 (1.28)
Bone	.277 (.273)	.035 (.036)	.028 (.028)	0.27 (0.26)
Plasma	145 (145)	104 (105)	5.1 (4.6)	

Table 26 is constructed in such a way that the data obtained in the "nephrotics" is outside the parentheses whereas the values of their appropriate controls are within the parentheses. The important findings were as follows: 1) There is a significant retention of sodium and chloride in muscle accompanied by a statistically insignificant loss of potassium; 2) There is an even greater retention of sodium and chloride in the skin, again with a statistically insignificant loss of potassium. 3) Tendon and bone showed no changes of any note; 4) There was a small but significant increase in the water content of muscle which agreed exactly with that which would be expected by theoretical calculations from the gain in sodium and chloride.

DR. FOX: How long after the edema appeared were the analyses performed?

DR. GRIBETZ: These rats were killed one to three days after the third injection. We were aiming for the day of maximum edema which usually occurred at this time.

DR. FOX: They were edematous, at the most, for three days.

DR. GRIBETZ: We never waited longer than three days for fear that we would miss the peak of the edema. Fortunately, we had a good rabbit and a rather "quick" disease was produced.

In the DOCA-treated animals, the following changes occurred (Table 27): 1) The plasma demonstrates the findings characteristic of a hypokaliemic, hypochloremic alkalosis; 2) Muscle shows a moderate retention of sodium and chloride, less than that of "nephrotics" accompanied by a significant decrease in potassium; 3) Tendon and bone, similar to the "nephrotics" showed no significant changes; 4) For some reason which is not clear to us at the moment, skin does not demonstrate any alterations in electrolyte, alterations which we might have expected from the changes in muscle and from the manner n which skin reacted in the "nephrotic" edema.

We saw no tissue changes in the first six animals studied after aldosterone administration. We apparently chose a dose which was too small to stimulate electrolyte alterations. The dose of 30 micrograms daily for five days was gleaned from the literature and from advice of people working with the substance but apparently it was not an optimum one. We should like to obtain some further advice on this.

TABLE 27

DOCA

	Na	Cl	K	H_2O
	mEq/100 g dry tissue			gm/gm dry tissue
Muscle	.089 (.077)	.057 (.050)	.353 (.410)	3.32 (3.09)
Skin	.181 (.183)	.166 (.174)	.063 (.065)	1.72 (1.82)
Tendon	.182 (.188)	.185 (.191)	.020 (.020)	1.27 (1.25)
Bone	.299 (.287)	.030 (.030)	.013 (.020)	0.26 (0.23)
Plasma	149 (142)	100 (106)	3.2 (4.0)	

DR. FOX: How many days of DOCA therapy were given prior to the tissue analysis?

DR. GRIBETZ: The DOCA-treated animals were all injected daily for seven days; they were sacrificed on the eighth day.

Figure 76 depicts graphically what you saw before statistically. Muscle demonstrated an approximate 33 per cent increase in sodium, 56 per cent increase in chloride

MUSCLE

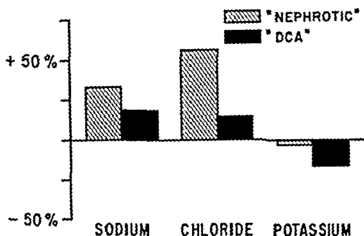


Fig. 76 Electrolyte composition of muscle following seven days of DOCA injection compared with composition of muscle in nephrotic rats.

SKIN

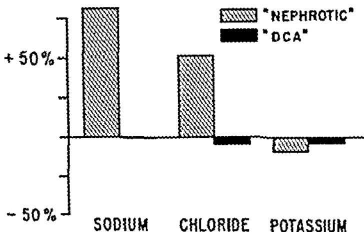


Fig. 77. Electrolyte composition of skin in rats treated with DOCA compared with nephrotic rats.

and a 3 per cent loss of potassium in the "nephrotics." The potassium loss, by statistical analysis, does not appear to be significant. In contrast, the muscle of the DOCA-treated animals had a 15 per cent increase in sodium, an approximate 14 per cent increase in chloride and a 16 per cent loss in potassium.

Figure 77 depicts the data for skin. In this tissue, the increases in sodium and chloride content were greater than in the muscles of the "nephrotics," 82 per cent for sodium and 51 per cent for chloride. There was an 8 per cent loss of potassium. As mentioned before, the skin of the DOCA-treated animals showed very little change.

On the basis of these findings, we have attempted some preliminary interpretations. If nothing else, they may at least stimulate some argument.

It is apparent that electrolyte from the dense connective tissues, such as bone and tendon, was not emptied into the extracellular space in response to the types of edema studied. This is in contrast to other stimuli such as acute acidosis.

Secondly, it appears as if the fluid and electrolyte derangement of at least "experimental nephrosis" in rats is characterized by a simple iso-osmotic expansion of the extracellular fluid. This fluid accumulates in those tissues which offer the least resistance to expansion, i.e., skin and muscle. The increase in muscle sodium and chloride spaces could be explained by a proportionate increase in extracellular volume. Calculations based upon chloride space produced almost perfect agreement between the observed increase in water content and that which was to be expected on the basis of the increase in sodium and chloride.

Thirdly, the absence of any unexplained change in water content in muscle plus the insignificant changes in potassium suggests that no complex intracellular derangements took place in the "experimental nephrosis."

Fourthly, the dense, non-yielding tissues such as tendon and bone, reflected very little expansion of the extracellular volume.

Finally, the tissues of the animals treated with DOCA showed a more marked fall in potassium with only a very slight expansion of sodium, chloride and water content.

We have therefore tentatively concluded that insofar as changes induced by DOCA administration typify those produced by aldosterone or other salt-retaining hormones of the adrenal glands, it is difficult to explain the edema of "experimental nephrosis" by assuming the etiologic influence of such hormones.

CHAIRMAN METCOFF: Thank you, Dr. Gribetz. I think, since some of the data I am going to present might be pertinent to discussion, perhaps I will present some of our material; then we can discuss both Dr. Gribetz' and our results following that.

E. Body Composition and Electrolyte Metabolism with Accumulating Edema and Diuresis in Nephrotic Rats

DR. METCOFF: Our interest in tissue composition was incidental to the problem of electrolyte transport occurring in both the experimental nephrotic syndrome and in children with the nephrotic syndrome.

The balance studies which had been done suggested that there was a large accumulation of sodium and chloride in nephrotic patients which could not be ascribed to any volume of distribution coincident with the increase in body weight. This suggested that the retention of sodium and chloride was two or three times as great as the gain in body weight, even if all the weight gain were attributed to accumulation of extracellular edema [6].

The French literature [7] suggested that there was something called a deposition of "dry" salt some place in the body during accumulating edema. This, from our physical-chemical knowledge, seems inconceivable; but it was stimulating and we thought it deserved further examination.

Moreover, the administration of different types of solute loads to nephrotic children, plus their behavior with various types of diuretic agents, suggested an unusual potential for removal of potassium, or some unusual potassium transport mechanism [8, 9]. This too seemed worthy of further study.

I would like to present some data today which are related to some of these objectives we have had. The data which I will show are based upon tissue analyses of nephrotic rats carried out in the period from 1954 to 1956, largely by Dr. Jorge Martner and Miss Irena Antonowicz in our laboratory.* Very young weanling male rats, 40-50 g. in body weight, derived from the Sprague-Dawley strain, were used. All rats were kept in individual cages and were pair-fed. All controls were pair-fed to the experimental animals.

The nephrotic syndrome was induced in weanling rats by subcutaneous injection of aminonucleoside [10, 11] after a short control period. The control period was

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It is apparent that electrolyte from the dense connective tissues, such as bone and tendon, was not emptied into the extracellular space in response to the types of edema studied. This is in contrast to other stimuli such as acute acidosis.

Secondly, it appears as if the fluid and electrolyte derangement of at least "experimental nephrosis" in rats is characterized by a simple iso-osmotic expansion of the extracellular fluid. This fluid accumulates in those tissues which offer the least resistance to expansion, i.e., skin and muscle. The increase in muscle sodium and chloride spaces could be explained by a proportionate increase in extracellular volume. Calculations based upon chloride space produced almost perfect agreement between the observed increase in water content and that which was to be expected on the basis of the increase in sodium and chloride.

Thirdly, the absence of any unexplained change in water content in muscle plus the insignificant changes in potassium suggests that no complex intracellular derangements took place in the "experimental nephrosis."

Fourthly, the dense, non-yielding tissues such as tendon and bone, reflected very little expansion of the extracellular volume.

Finally, the tissues of the animals treated with DOCA showed a more marked fall in potassium with only a very slight expansion of sodium, chloride and water content.

We have therefore tentatively concluded that insofar as changes induced by DOCA administration typify those produced by aldosterone or other salt-retaining hormones of the adrenal glands, it is difficult to explain the edema of "experimental nephrosis" by assuming the etiologic influence of such hormones.

CHAIRMAN METCOFF: Thank you, Dr. Gribetz. I think, since some of the data I am going to present might be pertinent to discussion, perhaps I will present some of our material; then we can discuss both Dr. Gribetz' and our results following that.

TABLE 29

DISTRIBUTION OF EDEMA IN NEPHROTIC RATS

Site	Per Cent of Total Edema
Ascitic fluid	67.6
Skin	16.7
Carcass	9.1
Organs	6.6

in the carcass and 21 per cent in the organs. Skin appears to be the major reservoir for edema fluid in rats[13]. This is similar to the distribution in children, as pointed out by Klose[14] many years ago. The changes in total body electrolyte and water content of edematous nephrotic versus control pair-fed rats over the 12 day period of accumulating edema is shown in Table 30.

TABLE 30

CHANGE IN TOTAL BODY ELECTROLYTE CONTENT AFTER 12 DAYS OF AMINONUCLEOSIDE INJECTION

Group		DFFS*	H_2O	Na	K	Cl
		g		mM		
A.	Initial Control**	8.64	40.5	2.3	2.9	1.6
B.	Control (13d)	15.68	49.7	3.0	4.6	2.3
C.	Nephrotic (13d)	13.81	81.8	5.4	4.3	4.4
D.	B-A	+ 7.04	+ 9.2	+0.7	+1.7	+0.7
E.	C-A	+ 5.17	+41.3	+3.1	+1.4	+2.8
F.	E-D	- 1.87	+32.1	+2.4	-0.3	+2.1

* DFFS - Dry Fat Free Solids
**Killed at start of experiment

Reference to line F of Table 30 indicates that the nephrotic rat gained less dry fat free solids and potassium and a disproportionately greater quantity of water than did the pair-fed control. The difference between electrolyte composition of the nephrotic and control rats was more obvious when related to a lean tissue or dry fat free solids reference (Table 31).

Since accumulating nephrotic edema appears to be associated with accumulation of less potassium and more sodium than expected, and since nephrotic children apparently

[13] Metcoff, J., Antonowicz, I., Frenk, S., Craig, J., Martner, J., and James, J., Skin as an electrolyte reservoir in the experimental nephrotic syndrome (abstract). J. Clin. Invest. 34: 953, 1955.

[14] Klose, E., Zur Kenntnis der Körperzusammensetzung bei Ernahrungsstörungen, Jahrb. f. Kindhlk 80. 154, 1914.

sufficiently long to establish a normal growth pattern. Several rats were sacrificed at the end of this period, prior to aminonucleoside injection, to determine a body composition base line. Aminonucleoside was injected daily into the remaining rats, some of which were placed on balance study. After 12 days of such injection, when the nephrotic syndrome was well established, the animals were killed. By comparing the electrolyte and water composition of these rats and their pair-fed, non-nephrotic controls with the control rats killed 12 days earlier the extent and distribution of edema was measured, and comparison of electrolyte apposition by direct analysis with that estimated by balance measurement was possible.

Muscle, skin, organs and residual carcass were analyzed. Bone and tendon (except for tail) were included with the carcass analysis. Our initial studies of edematous rat skin in 1953 indicated that the location of the biopsy and the quantity of skin obtained produced marked variation in the data. To overcome this problem total skin was analyzed. Under nembutal anesthesia, hair was removed by the technique of Walser and Bodenlos [12], then the skin was removed from the animal (after death by exsanguination) like stripping a glove off one's hand. The final data for body composition represents a tabulation of the individual analyses for muscle, skin, organs, carcass, blood (and ascitic fluid in edematous animals). By comparing weights of the intact, anesthetized, depilated rat with summed weights of all the parts subjected to analyses, it was evident that our losses in the analytic preparatory procedures did not exceed 1 to 2 per cent.

We were not particularly confident of our balance technique in these rats, there may have been some unappreciated urine losses or diet contamination of excreta despite all our precautions. Table 28 shows the average increment in total body Na, K and Cl of nephrotic rats compared to their pair-fed controls as revealed by direct analyses and a similar comparison of the same animals based on balance studies. It is evident that the balance technique is not quantitatively reliable.

TABLE 28

COMPARISON OF TOTAL ELECTROLYTE INCREMENTS IN NEPHROTIC RATS VS. CONTROLS BY (a) DIRECT ANALYSIS AND (b) BALANCE MEASUREMENTS

	Na	K mM	Cl
(a) Direct analysis	+4.17	-0.32	+3.46
(b) Balance	+5.11	+2.35	+2.83

The distribution of the accumulated edema in 18 nephrotic rats is given in Table 29. Most of the water accumulation occurs within the abdominal cavity and therefore has the composition of extracellular fluid. However, excluding this free fluid, approximately 51 per cent of the total tissue edema was residual in the skin, 28 per cent was found

[12] Walser, M. and Bodenlos, L. J., Composition of skin as compared with muscle. Amer. J. Physiol., 178: 91, 1954.

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E.	C-A	+ 5.17	+41.3	+3.1	+1.4	+2.8
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* DFFS - Dry Fat Free Solids
**Killed at start of experiment

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[14] Klose, E., Zur Kenntnis der Körperzusammensetzung bei Ernahrungsstörungen, Jahrb. f. Kindhlk 80: 154, 1914.

TABLE 31

CHANGE IN BODY ELECTROLYTE CONTENT BASED ON INCREMENT OF TISSUE

	H_2O	Na	K	Cl
	g	μM		
	Increment Per Gram Lean Tissue* Gain			
Nephrotics	0.89	67	30	60
Controls	0.57	43	105	43
	Increment Per Gram DFFS Gain			
Nephrotics	8.0	600	271	540
Controls	1.3	99	242	99

* Lean tissue gain equal DFFS plus H_2O gain over initial control values. (Line A, Table 30)

have some alteration in the renal tubular potassium transport mechanism[8, 9], it seemed of interest to determine the change in total body and specific cell composition in response to a challenge posed by omitting sodium intake and restricting carbonic anhydrase activity after marked edema had been achieved by sodium loading and while the nephrotogenic chemical was still being injected. The results of this experiment are shown in Table 32.

TABLE 32

EFFECT OF SODIUM FREE DIET AND DIAMOX ON CHANGE IN BODY COMPOSITION*

	Δ DFFS*	H_2O	Na	K	Cl
	g	g	mM		
			(Per g. DFFS)		
Controls	+2.90	+ 1.7	0	+0.38	0
Nephrotic	+0.70	-30.7	-0.3	-0.4	-0.1

*From the 13th to 18th day of aminonucleoside injection.

All of the edematous nephrotic rats diuresed on this regimen. In the controls, an expected gain of 0.9 mM K (cf. Table 31) was not achieved. Since there was no sodium intake or change in body sodium content, the lack of exogenous sodium presumably affords a sufficient stimulus to conserve body sodium maximally despite blockade of the hydrogen ion exchanging mechanism. Since less potassium was retained in the new protoplasm laid down, it seems likely that final sodium reabsorption in the renal tubule occurred via potassium exchange. During the six day interval, the diuresing nephrotic rats gained about one quarter as much dry fat-free solids as the pair-fed controls.

Inhibition of carbonic anhydrase activity, possibly implemented by the sodium free diet in nephrotic rats, induced large excretions of both sodium and potassium.

The estimated urinary excretions of Na, K and Cl based on the difference between measured dietary intake of these items and change in analyzed body composition is shown in Table 33.

TABLE 33

ESTIMATED URINARY EXCRETION (INTAKE - $\triangle$ BODY COMPOSITION)

	Na	K	Cl
		mM	
a) During Accumulating Edema (12 days)			
Nephrotics	6.9	13.6	8.9
Controls	12.6	12.0	13.1
b) During Diuresis (6 days)			
Nephrotics	6.7	10.7	8.5
Controls	1.0	9.1	3.9

The alteration in cell composition resulting from diuresis under the above circumstances are more difficult to interpret. The extracellular phase expansion in the nephrotic rats indicated that the diuresis was incomplete (Table 34).

TABLE 34

MUSCLE COMPOSITION FOLLOWING DIURESIS WITH DIAMOX AND SODIUM FREE DIET

	ECW	ICW	$(Na)_e$	$[Na]_i$	$[K]_i$	$(K)_i$/NcN
	g	g	mM	mM/L		mM/g
	Per 100 g DFFS					
Controls	59	262	9.1	1.8	178.0	3.72
Nephrotics	84	267	11.8	18.9	154.0	3.12

Sodium concentration in the ECW of the nephrotic rats was lower than that of controls. The [Na + K] concentrations intracellularly were also lower than those of the controls. The effect of the carbonic anhydrase inhibitor appears to be different in muscle than in renal tubular cells in the control rats in the absence of dietary sodium. Cell sodium concentration decreased and potassium concentration increased. An increment of hydrogen ion equivalent to the 3 + mM/L ICW sodium lost can hardly explain an accumulation of potassium of nearly 20 mM/L ICW during the diamox, sodium-free diet phase. However, the relative intake per gram of body weight gain was much greater during the latter interval.

The concentrations of both intracellular sodium and potassium decreased by 9 and 12 mM/L ICW, respectively, in the nephrotic rats during diuresis. The loss of potassium from cells is contrary to usual experience with diuresis in nephrotic children[15]. Carbonic anhydrase inhibition seems the most likely explanation. If so, then transport of Na and K out of the cells would be independent phenomena, possibly related to presumed accumulation of hydrogen ion intracellularly.

A final study details the effect of diet electrolyte restriction during development of the nephrotic syndrome in weanling rats. In general, three diets were used: our usual control diet; one with low sodium plus substituted organic cation, and one with low sodium but completely potassium-free. All animals were pair-fed against the aminonucleoside-injected, potassium-free diet rats, hence all suffered relative caloric and protein malnutrition from inadequate intake.

Although calcium, magnesium and bicarbonate concentration of the ECW were not determined, it is possible to estimate the relative changes in bicarbonate plus organic acids based on the change in concentrations of sodium, potassium, chloride and protein in the various groups. In the control diet groups, nephrotic and control rats had 31-33 mM/L more cations (Na + K)-(Cl + protein). In the low sodium - no potassium groups the difference was augmented by 9-10 mM/L, suggesting an increase in the bicarbonate plus organic acids fractions. It seems probable that most of this increment was contributed by bicarbonate, and probably represented extracellular alkalosis characteristic of potassium depletion.

The alterations in cell composition associated with these dietary challenges are shown in Table 35.

TABLE 35

MUSCLE COMPOSITION
(Per 100 g DFFS)

	Diet	Group	No. Rats	H_2O (g)	NcN (g)	Na (mM)	K (mM)	Cl (mM)
I.	(Control)	Control	5	341	13.4	16.9	32.3	7.0
		Nephrotic	5	383	13.9	19.6	37.9	11.5
II.	(Low Na)	Control	9	336	13.6	18.6	35.6	7.3
		Nephrotic	7	389	13.6	20.3	37.1	13.2
III.	(Low Na-No K)	Control	7	349	13.2	23.2	27.6	7.7
		Nephrotic	4	426	13.0	23.1	34.2	13.6
IV.	(Control ad lib. fed)		6	340	13.0	13.0	41.1	6.5

[15] Metcoff, J. and James, J., Fourth annual conference on the Nephrotic Syndrome, p. 109, 1952.

It is noteworthy that the greatest deviations occur in the potassium-free diet group. The limited availability of sodium in the diet, as well, led to maximal conservation of this ion. Note that cellular potassium depletion was greater in the non-nephrotic animal, and could not be related to greater sodium retention. It is possible that the increased cellular catabolism of the nephrotic rat yielded a better endogenous source of potassium in support of surviving cells. However, the diet potassium-deficient nephrotic rats accumulated water out of proportion to their relative retention of electrolyte.

The water and electrolyte contents in muscle of young rats allowed ad lib. consumption of the basic diet are presented (Group IV) for comparison.

The intracellular concentrations of sodium and potassium observed in the various diet groups are given in Table 36. The concentrations of Na are high and of potassium low in all the non-nephrotic rats. It is interesting that the low sodium diet controls showed slightly elevated intracellular Na concentrations. The reduced concentrations of cellular potassium may reflect the fact that all animals were malnourished.

TABLE 36

CELLULAR CONCENTRATIONS OF Na AND K (MUSCLE)

Diet	Group	ECW	ICW	$[Na]_i$	$[K]_i$	K_i/NcN
		g/100 g DFFS		mM/L ICW		mM/g
I	Control	58	282	24	126	2.6
	Nephrotic	95	288	16	129	2.6
II	Control	63	273	29	122	2.5
	Nephrotic	107	282	14	129	2.6
III	Control	70	279	43	99	2.1
	Nephrotic	117	309	23	111	2.6

The greatest distortion in cellular composition appeared in the K deficient, low sodium diet control rats. The greatest accumulation of Na and loss of K from cells occurred in this group. There was no significant change in total phosphate of the cell. The accumulation of sodium intracellularly during K depletion apparently does not depend upon excess Na intake. Similarly, intracellular accumulation of Na under the conditions of this experiment was not associated with expansion of cell volume. The nephrotic rats exhibited expansion of extracellular volume despite sodium intake restriction, however, expansion of both extra- and intracellular volumes was most marked when no exogenous K was available, despite low sodium intake. It was of particular interest that the cellular Na concentrations of all nephrotic rats were lower than in pair-fed controls. Since cellular phosphate was not altered, and it was unlikely that cell magnesium was increased, the additional cation might be derived from either specific amino acid or hydrogen ion accumulation.

Quantitative data for amino acid values in muscle of rats were not available for comparison, but Dr. Elizabeth Kaiser in our laboratories has extracted the dry fat free

muscle solids and chromatogrammed and quantitated the extract. Quantitation was achieved with a Spinco "Analytrol." The results are shown in Table 37.

TABLE 37

MUSCLE AMINO ACIDS
(mg α amino N/100 g DFFS)

Diet	Group	No. Rats	Glutamic	Lysine	Arginine	Alanine
I	Control	6	0.1-0.7	0.8-2.6	0.2-1.0	0.6-2.1
	Nephrotic	1	0.2	0.8	0.4	0.7
II	Control	5	0.1-0.8	0.7-1.2	0.2-0.6	0.7-1.3
	Nephrotic	4	0.3-0.8	0.2-2.6	0.3-0.8	0.1-2.3
III	Control	2	0.1-1.2	1.0-1.2	0.3-0.9	1.1-1.5
	Nephrotic	1	0.2	1.8	0.7	1.3
IV	Control (ad lib. fed)	5	0.1-0.6	0.5-1.0	0.4-0.7	1.2-2.0

Unfortunately, we did not have enough tissue for analysis in all groups, and therefore no definite conclusions can be drawn. The range of values, however, suggest that cationic amino-acid accumulation (Lysine) could not account for the cation discrepancy. If this is true, then the nephrotic syndrome in K depleted rats is associated with accumulation of hydrogen ion and water intracellularly - a kind of acidophilic swelling.

Changes in composition of the total skin were of interest in that the increment of chloride was relatively greater than that of sodium in the edematous skins (Table 38). There was a loss of K from the skins of potassium-depleted rats. There were no noteworthy alterations in collagen or non-collagen nitrogen. The water content of the edematous skin was not as great as that previously reported[11]. This deviation may have resulted from dietary deficits. Note that edematous rats on low sodium intakes accumulated as much sodium and H_2O as did those on more adequate sodium diets.

Finally, a comment on total body analysis which seems directly applicable to clinical experience. The apparent wasting of the tissues of nephrotic children noted on diuresis after a protracted siege of edema has always suggested that tissue catabolism was a constant feature of the nephrotic syndrome and contributed to the dynamic equilibrium of the plasma protein pool, which in turn was depleted by both proteinuria and catabolism. Balance studies suggested a simultaneous depletion of body potassium[15], but this was not apparent from studies with exchangeable K^{42}[16]. The data of Table 39

[16] Gribetz, D., Corsa, L., Cook, C. D., Keitel, H., and Talbot, N.B., Measurement of total body exchangeable potassium and erythrocyte potassium in nephrotic children. J. Clin. Invest. 33: 680, 1954.

TABLE 38

TOTAL SKIN CONTENT
(Per 100 g DFFS)

Diet	Group	No. Rats	Na	K	Cl	H_2O
			mM			g
I	Control	6	28.9	19.6	22.8	316
	Nephrotic	5	33.9	17.2	29.1	344
II	Control	9	28.1	18.0	21.7	297
	Nephrotic	9	36.7	18.0	31.4	361
III	Control	7	28.7	14.6	20.5	296
	Nephrotic	4	32.2	15.7	26.8	320

indicate either failure of apposition, or loss of total body dry fat-free solids, by nephrotic rats, relative to pair-fed controls. While the potassium and non-collagenous nitrogen of the nephrotic animals was markedly reduced, the potassium content of the residual dry fat free tissue of the nephrotic animals was equivalent to or greater than that of the controls. The non-collagenous nitrogen of the surviving tissue of the nephrotic rats was reduced slightly.

The potassium content per unit of protoplasmic nitrogen (K/NcN) was slightly increased in the electrolyte depleted groups. The reason for this is not clear. Thus it would appear that the early phases of the nephrotic syndrome in growing rats are associated with failure to gain or loss of quanta of protoplasm, but that the electrolyte anatomy of surviving cells is essentially normal. This may result from endogenous utilization of K and N derived from catabolic processes, as exemplified by the loss of nitrogen but preservation of the potassium ratios in the rats on potassium-free diets.

TABLE 39

TOTAL BODY COMPOSITION

Diet	Group	DFFS	NcN/DFFS	K/DFFS	K/NcN
		g	g/g	mM/g	
I	Control	11.4	0.088	0.291	3.32
	Nephrotic	9.7	0.088	0.288	3.28
II	Control	11.4	0.086	0.266	3.09
	Nephrotic	9.6	0.082	0.294	3.57
III	Control	9.2	0.085	0.263	3.10
	Nephrotic	7.8	0.079	0.272	3.42

Thank you. Both the preceding reports are now open for discussion. Dr. Barnett!

DR. BARNETT: Dr. Gribetz, would not an adrenalectomized rat made edematous by giving DOCA provide a better control for the nephrotic rat?

DR. GRIBETZ: I think it probably would; however, it would be much more trouble. (Laughter)

DR. BARNETT: Well, I do not know. I think this is a very interesting approach of trying to elucidate the possible role of the adrenal glands in the pathogenesis of edema in the nephrotic animal, but it is not very convincing when the animal treated with DOCA does not have edema.

DR. GRIBETZ: As endocrinologists, we would agree that it is not as convincing. Wherever there is endogenous secretion of an endocrine gland, it is difficult to separate the effects of this from those of the artificially administered hormone.

In presenting our data, I made a grievous omission. I neglected to state that these studies were performed in conjunction with Dr. Marvin F. Levitt; in fact, he was the stimulus for the project. Perhaps he would like to comment on the question.

DR. LEVITT: It is not fair to say that within five days, animals rendered nephrotic accumulate a considerable amount of extracellular expansion and, within a comparable period of time, those given large doses of DOCA do not retain a comparable degree of sodium.

I think Donald was a little humble with the aldosterone data, too. He did give perfectly good doses of aldosterone, presuming that the ratio of activity was about 100 to 1. Despite the administration of aldosterone for a period of six to seven days, there was absolutely no salt retention detectable by skin or muscle analysis. I think it is something which has to be considered in terms of trying to attribute the edema of the nephrotic syndrome to the role of aldosterone or DOCA.

DR. BAXTER: Isn't this another case of the "added factor" which was discussed a little while ago; that one group of rats has a low serum albumin, and the other has a normal serum albumin.

It seems to me that serum albumin level may explain the differences between the nephrotic rats and those receiving DOCA or aldosterone.

DR. GRIBETZ: Actually, the group of rats with the lowest serum albumin, the nephrotic rats, were the ones which showed the greatest retention of the sodium chloride.

DR. JAMES GAMBLE, JR.: We did some studies quite some time ago with only two relatively new nephrotic patients, in whom we measured the combined distribution of sodium-24 and chloride-36 which is rather a difficult determination to make.

In the new experiment, the ratio was close to our normal value; we got a ratio of about 1.05.

However, we also did some measurements in the terminal cases; and, in these, the sodium space was very greatly enlarged relative to the chloride space. In fact, this was on a reasonably high protein intake; I do not remember the figures right now. If we lowered the protein intake, the sodium space would shrink down toward the chloride space.

It might be that, in the fresh nephrotic, there is relatively little adrenal activity; in the terminal stage, three or four months from death, there would be more adrenal activity, which might reconcile some of these points of view.

DR. LEVITT: You could also interpret that by saying the terminal patient has been subjected to considerable K loss with replacement by sodium. The nephrotic, who has not been eating, and who has been given many diuretics, is more likely to be K-depleted than is the untreated nephrotic.

DR. FOX: I wonder if anybody has any data on tissue analysis in humans at various stages.

Some years ago, we did some studies of the muscle and skin and other tissues in patients. I think we have to recognize the fact that, in Dr. Gribetz' experiments, the animals had only been edematous for three days. Jack, yours were edematous for twelve days.

We know the patients are edematous for a considerably longer period of time; and I think all our knowledge indicates that time is an important factor here. In the work we did, there was a significant reduction in potassium per unit of dry weight, and a rather large increase in sodium, disproportionate to the water content. So I think there was some evidence of an exchange. Whether this was due to the ill health of the patient - as you mentioned, Marvin, they may have been potassium-depleted - or whether this is an intrinsic part of the disease, I think remains open to question. I think it might be of value to try to obtain data in patients we see from time to time, perhaps, in another year or two, we might get some data concerning the human disease at different stages.

By the way, Dr. Gribetz, you did have some interesting data on exchangeable potassium in some nephrotic children. I suggest that you include the calculations for the growth of these children. As I recall, the children who grew did not put on potassium nearly in proportion to their weight, as you would anticipate. Of course, this is highly speculative, but --

DR. GRIBETZ: That apparently was true. Those studies [16] showed that there was no depletion of potassium during the stage of the disease that we studied. But, of course, exchangeable potassium methods are gross measurements at best.

DR. FOX: You did some of these patients two or three times, with time lapsing between determinations. There was no increase in their exchangeable K over the period of months which lapsed in some cases. Therefore these children were stagnant in terms of K during the period of growth.

CHAIRMAN METCOFF: In regard to "exchange" of sodium and potassium, I was rather hoping that someone would comment to the effect that, there was no evidence in our rat tissues of an "exchange" of sodium and potassium. That is, Na and K may vary quite independently of each other, intracellularly. As Dr. Fox suggests, our rat data are consistent with the idea that less than expected increments of K occur with accumulating edema during a growth period.

DR. FOX: Did your animals have an increase of aldosterone?

CHAIRMAN METCOFF: I am sure I do not know.

DR. GRIBETZ: Has anyone else given aldosterone to rats; and could he give us some idea of what the proper dosage is?

DR. GRIBETZ: We used Cole's paper[17] as a guide. They suggested that a 250 gm. rat has an output of 0.75 micrograms of aldosterone per hour and thus 10 micrograms would be at least a 10 hour supply. We thought our dose of 30 micrograms per day in 100 gm. animals would be sufficient to produce changes. Because our animals were not adrenalectomized, perhaps they require much larger doses.

DR. HOLLIDAY: Dr. Metcoff, why do you think your rats, both control and nephrotic, had such low potassiums in relation to water? Those are really quite low values.

CHAIRMAN METCOFF: I do not know, possibly because of some malnutrition since they were pair-fed. They had high intracellular waters too, as you noted.

DR. HOLLIDAY: Was their serum sodium below normal?

CHAIRMAN METCOFF: No.

DR. HOLLIDAY: Perhaps their potassium intake also was deficient.

CHAIRMAN METCOFF: Yes; and this may have been a function of their malnutrition. This might account for increased intracellular water in relation to that observed in the ad lib. fed rats.

If there are no further comments, I think we can adjourn. The meeting tomorrow morning will start at 9 a.m.

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[17] Cole, D. F., Effect of aldosterone on renal excretion of intravenously administered saline, Endoc. 60: 562, 1957.

SATURDAY MORNING SESSION

October 26, 1957

The meeting reconvened at nine-fifteen o'clock, Dr. Barnett presiding.

CHAIRMAN BARNETT: We plan this morning to discuss what people are doing currently in the laboratory or in the form of clinical investigation; and, secondly, to discuss (which is really part of the same thing) that phase of clinical investigation which relates to treatment.

These presentations, which are informal and do not have the elegance of the prepared presentations which preceded them, have been extremely useful, I think, to all of us. They have served the purpose of bringing to current problems the suggestions and criticisms of other people working in similar, related fields.

Although Jack has been working pretty hard at this conference, I would like to ask him to begin.

IV. Work in Progress

A. Dr. Jack Metcoff

DR. METCOFF: Before I start, I would like to thank and compliment our hosts on the magnificent job which they have done in organizing the conference and providing all of us with a very stimulating session. I think we have all enjoyed it; I believe that we have learned a great deal. We are all deeply appreciative of the continued support of the National Nephrosis Foundation, which makes transcription and a permanent record of these proceedings available to all. We have had to limit the attendance to active investigators in order to keep the group small, so that we can maintain a kind of informality, with emphasis on discussion of new material being presented.

Our own laboratories' current investigations pertain to the problems of transport and energy limitations in transport, in tissues, with particular reference to terminal carbohydrate metabolism.

There appear to be low potassium concentrations and increased water content within the muscle cell of nephrotic rats. We do not know the significance of this.

We are particularly concerned with one of the final steps, where phosphoenol pyruvate is transformed into pyruvate, and ADP to ATP. This step is catalyzed by

pyruvic phosphoferrase and magnesium is essential. This particular transfer is one of the few which is K-dependent.

We have been particularly concerned with the general components of the Krebs cycle. In addition to pyruvate we have been measuring alpha-keto glutarate, and attempting to measure oxalacetate. We are trying to determine whether alterations in the water, sodium and potassium content of the cell will alter the rate of production of these metabolic end products.

We have been carrying out similar studies in protein malnutrition.

Another type of experient going on may be of some interest. We are currently in the process of dialyzing dogs in order to induce marked hypotonicity. We produce a change in osmolarity of the ECF from 310 to 260 mosm/L in about two hours.

During the course of the dialysis, we measure renal functions, and obtain muscle biopsies at the start and again at the end of dialysis.

By this technique, we hope to learn something about the renal adjustments to the development of the acute hypotonicity and the role of cellular reorganization and function in relation to these renal adjustments.

We are also continuing to do amino-acid analyses of muscle, and think we have our system fairly well organized to do this, hoping that we may be able to get further information concerning the relation of these components of protoplasm to electrolyte metabolism.

CHAIRMAN BARNETT: Thank you, Jack.

Are there any comments on what Jack has described?

DR. NORMAN KRETCHMER (Department of Pediatrics, New York Hospital): I would just like to ask a question, Jack. Are you measuring just the phosphoenol pyruvate to pyruvate, or the whole Krebs' scheme?

DR. METCOFF: We are trying to pick up the whole Krebs' cycle, Norman; but we are at the moment measuring three points on it: pyruvate, alpha-keto-glutarate, and oxalacetate.

DR. KRETCHMER: You are then making your measurements in the presence of oxygen?

DR. METCOFF: Yes.

DR. KRETCHMER: Thank you.

CHAIRMAN BARNETT: Would you like to comment on your question? (Laughter)

DR. KRETCHMER: Yes, if I might discuss my question. It would be enzymatically more feasible to do the experiment in the presence of nitrogen, and measure just the one step, phosphoenol pyruvate to pyruvate. In that way, you would not become involved with at least eight or nine enzymatic steps; and the reaction where, presumptively, potassium was participating would be isolated.

DR. METCOFF: Isn't the shift from pyruvate to lactate extremely rapid and difficult to isolate?

DR. KRETCHMER: Yes, but the hydrogenation system can be blocked. What you are doing is just adding one molecule of hydrogen to go to lactate. You can actually trap the hydrogen and it might make it simpler to measure pyruvate colorimetrically.

DR. METCOFF: Thank you, Norman, that seems like an excellent suggestion.

I would appreciate any other comments.

If anybody has already done this, or is in the process of doing it, please let me know; then we will not have to do it.

CHAIRMAN BARNETT: Walter, would you --

B. Dr. Walter Heymann

DR. HEYMANN: I do not want to take much time because I would like Sidney Wilson to report on the work he is doing concerning the aminonucleoside disease which is, we believe, a fascinating problem. There may be some value in reporting studies, even though the results were negative. It might save someone else the work.

Because the detection of renal antibodies in sera of nephrotic patients has failed to yield positive results, the possibility has to be considered that these substances are most efficiently cleared from the blood stream and fixed in renal tissue. It does seem justified to try to develop a method to detect them in kidney material.

When we inject rats with a very potent anti-kidney serum, we know from Pressman's work that heteronephrotoxic antibodies are cleared from the blood stream within 11 minutes and localized chiefly in the glomerular structures. We, thus, obtained glomerular suspensions from kidneys of rats that had been injected with anti-kidney serum two to four days previously. These suspensions contained glomeruli with and without capsules. They were brought in contact, for varied lengths of time, with tannic acid treated sheep red blood cells that had been coated with rat kidney antigen. No agglutinations of these red blood cells along the surface of the glomeruli were noted. Immunologists do consider monovalent and bivalent antibodies. The reason for the failure of surface agglutination may be that heteronephrotoxic antibodies are monovalent.

We then have tried to elute the antibodies from nephrotic rat kidneys, and to use the eluates for hemagglutination procedures. We did apply acidification and heat, 60° for half an hour, for elution. There were three out of 12 such experiments in which the eluates gave some titers. Nine, however, remained negative. We still continue this work, but there certainly is no easy way to elute antibodies from kidney tissues.

DR. RAPOPORT: Why can't you do this - I am naive and a complete amateur about this.

DR. HEYMANN: I, too.

DR. RAPOPORT: I have a vague recollection of a reading contact. Is there not a migratory starch column in which there are antigens of different kinds, and in which a labelled eluate migrates according to its affinity for antigen?

Someplace, somebody has studied antibodies which can be differentiated only by migration in starch columns, which differ only in the antigen. In such a system you can pick up differences which you cannot pick up by any other known immunologic setup. It is a kind of column chromatography.

DR. LANGE: There is a technique of elution with salicylate, with quite a high yield from the eluate of kidney from a rat with nephrotoxic nephritis. We have tried the same technique, but so far, we have been unsuccessful.

DR. HEYMANN: Yes. I discussed these procedures at the time with Dr. Pillemer; he was not very impressed.

DR. LANGE: You have not succeeded in repeating it?

DR. HEYMANN: We have not tried it. I would like to say that it appears to be a good model to work with.

DR. PFEIFFER: I would like to return to Dr. Heymann's remark. When you have the kidney glomeruli isolated, then you have made an important step forward. We assume that we are dealing with an immunological process in glomerulonephritis. You have isolated antigenic material and your hypothetical antibodies can be bound by their antigens.

The next step - I suppose this was why you studied this preparation - would be to put some rabbit antiserum against rat serum on slides with your glomerular suspensions. Then you should be able to detect, as with the Coombs' test in hematology, incomplete antibodies which may play an important role in our problem. The whole hypothesis of auto-immunization in hematology came up only after the discovery of this technique. In the past, I am sure you know, nobody had demonstrated the auto-antibodies with reliable methods, but now it is established that the auto-immune-mechanism is involved in many blood disorders.

DR. HEYMANN: We have tried that with the addition of various proteins, but without success. We have, however, not had the anti-gamma globulin as yet, which we should have used.

DR. PFEIFFER. Another point I would like to mention is that we have tried to demonstrate antibodies against kidney tissue antigens also with the tannic-acid erythrocyte technique after the modification of Stavitsky. We have not been too successful with this method in man. However, in rats with experimental nephritis, we got positive

results with this technique only after ten days of nephritis. Then the titers were fairly reproducible, and the controls were entirely negative.

When we started this project, however, we intended to perform some kind of blocking test. That means, if the sensitized erythrocytes are completely occupied by auto-antibodies against kidney, no agglutination will occur when you add an anti-kidney antiserum from another animal to the slide; but, if no auto-antibodies are present, agglutination will be visible.

However, because we already had positive results, presumably due to complete antibody-like factors before the addition of a nephrotoxic serum, this mechanism did not work.

With this blocking method, therefore, you can look for negative agglutinations as positive reactions and interpret positive agglutinations as negative indicating that no antibody in the test-serum has "blocked" the antigenic receptors on the surface of the erythrocyte.

DR. HEYMANN: Yes.

DR. PFEIFFER: In man, I would suggest we should try this because we already had positive results with the simple technique in animals.

DR. HEYMANN: Yes, that is right; that is published. That works quite well.

DR. CHARLES A. JANEWAY (Harvard Medical School). Walter, have you tried a suspension of glomeruli from a normal rat kidney in vitro with your nephrotoxic serum?

It seems to me one of the things you have to do is create a situation in which the binding groups of the antibodies are not all fastened down, so to speak. You could achieve this in an in vitro system and never know it; I mean you just do not have any idea how much serum it takes in the animal to exceed the capacity of the kidney to absorb antibody.

DR. HEYMANN: If one incubates a glomerular suspension obtained from normal rat kidneys with nephrotoxic serum, one detoxifies it rapidly and an antigen-antibody junction takes place. The nephrotoxicity of the serum will be decreased or possibly abolished.

DR. LANGE: Maybe, if you would do it in a complement-free medium, so that the union does not take place and there is no cell destruction, it may work.

DR. HEYMANN: May I comment about something which is silly most probably. (Laughter) For years, I thought maybe one should see whether there is an intracutaneous response to kidney antigen in nephrotic patients. I do not know whether anybody had ever done this experiment.

DR. JANEWAY: We did, with negative results.

DR. HEYMANN: We have done it, with negative results. You did, too. Good!

As far as the hyperlipemia problem is concerned, I also have only a little to report. You may remember that Rosenman, Byers and Friedman have proposed the hypothesis that hyperlipemia occurs in the nephrotic syndrome because lipids are trapped in the plasma, the liver being unable to take up plasma-lipids properly.

Together with Abe Bergman and Dr. LeRoy Matthews, we have injected rats with C_{14} trilaurin emulsions and have followed the clearance of this fatty acid from the blood, and the up-take of this labelled compound in liver and kidneys. I can summarize the results of these studies by saying that the nephrotic animals behaved in an indistinguishable fashion from the controls. The up-take in the livers of nephrotic rats, in particular, was at least identical, if not superior, to the up-take obtained in non-nephrotic rats.

Thus, as far as trilaurin is concerned, there is no support for the aforementioned hypothesis.

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DR. HEYMANN: Yes.

DR. RAPOPORT: And this high level may be the driving pump which moves fat across the cell membrane. This is all speculation; but, if you have a nephrotic animal whose blood you have cleared of fat, and then introduce fat at different levels, and equilibrate it at, say, 100 mg., 200 mg., 300 mg., you might then be able to show that you need a level of 400 mg. before fat enters cells at a normal rate, and that this might be the explanation for the trapping.

DR. HEYMANN: It is interesting to point out that the nephrotic hyperlipemia that is so impressive in terms of milligrams per cent plasma, amounts to approximately only 30 to 90 milligrams of total lipids when calculated as surplus milligrams of total lipids per plasma volume of a 200 gram nephrotic rat. Do you think that this would alter your way of thinking?

DR. RAPOPORT: No, this is analogous to the fish, which is uremic. It maintains a high blood level of urea to clear the small amounts it has to every day. Of course, its filtration is always a basal 300 mg., plus the additional 20. It gets rid of these 20 milligrams in a small volume of fluid; and it does not require very much change in concentration.

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We have seen recently a little girl who had been treated with tridione for petit mal, for a period of eight months. She developed the nephrotic syndrome so severely that we almost lost her. I became interested in whether this could be experimentally reproduced in rats; according to the literature, this has not been successful[1]. I was

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not discouraged by this report because tridione had not been given over a period of time which could compare with those observed in children who take tridione and develop the nephrotic syndrome after usually 6, 7 or 8 months of medication.

Thus, we have tubed rats with tridione day by day for up to 13 months. It usually takes approximately 6 months for rats to develop the disease, and only 9 of 20 animals have, thus far, developed a nephrotic syndrome.

It is not a fulminating disease like the one that we produce with potent nephrotoxic sera. The tridione-treated rats develop proteinuria of moderate degree with 200 to 300 milligrams of protein excreted per day; they have a moderate hypoproteinemia and hyperlipemia without azotemia or hematuria. By the time they develop renal disease they are at least 7 to 8 months old and anasarca has not been observed as yet. Renal failure has not been noted as yet, either. Experimentally, a mild nephrotic syndrome thus can be produced in rats with tridione, if one keeps on giving it over long periods of time.

DR. GRIBETZ: What dose did you use?

DR. HEYMANN: We have used about five to ten times the dose which is used in epileptic children. The rats were tubed with 1.0 to 1.25 cc. of the solution, which is approximately 40 mg. per day.

CHAIRMAN BARNET: You start with young rats.

DR. HEYMANN: At the beginning the rats weighed 100 to 150 gm.

CHAIRMAN BARNET: Dr. Wilson!

C. Dr. Sidney Wilson

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If it is given in the dose which he described, which was 0.3 ml. of a ½ per cent solution per 100 gm., proteinuria appears in 7 to 13 days. We usually continue the injections for 12 to 14 days. Proteinuria will persist for a variable length of time from about 3 weeks to 2 months, then disappears.

This latent period interested us considerably, because it could be reduced or lengthened, according to the dose. If we reduced the daily dose, the latent period was

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much more severely ill; and many of them died. But those control rats which had irradiation *alone had no proteinuria, and survived* for a month without any disease.

Further investigating whether it was caused by an antibody, complement titers at the onset and during the course of the disease were measured. Here we have come up against something which does not fit in with what we have said before.

Out of 8 rats in which we measured complement, we found, in 6 of them, that there was a depletion of complement at just about the time when proteinuria first appeared. This might be interpreted by many as being very strong evidence in favor of an antigen-antibody reaction being the cause of the disease.

But we are not very sure that this evidence of depletion of complement in these rats is necessarily indicative of an antigen-antibody reaction occurring in this particular disease.

We have also looked for antibodies in the serum by the hemagglutination technique, and have drawn a complete blank there. We did not find any positive results by the hemagglutination method.

CHAIRMAN BARNETT: Has complement been measured in the rats which were irradiated or treated with steroids?

DR. WILSON: No.

DR. JANEWAY. I think that complement has to be evaluated in relation to observations Dave Gitlin and other people have made on protein catabolism. Complement is a substance which, at least in considerable part, is protein; and there are forces operating which lower the serum levels of practically all the proteins. Complement shares in this, although it is a difficult one to measure in its individual components.

DR. LANGE. Complement usually does not go down as severely.

DR. JANEWAY. Neither do you have the same phenomena operating as in nephrosis where, for some reason, you have increased catabolism of many of the proteins.

DR. HEYMANN: This aminonucleoside is a toxic substance which, outside of producing renal disease also has some other effects.

DR. LANGE: May I suggest a simple experiment? Take the blood of animals which have had proteinuria for 5 days, and transfer it to an animal to which you have just given the substance, and see whether he gets an immediate disease.

DR. HEYMANN. Similar to what you did with the rabbits injected with duck serum. That is a good idea, indeed.

DR. LANGE: It may be very good proof that you have an antibody, just passive transfer of antibody from a diseased animal to a sensitized animal.

lengthened to something like 14 or 17 days; and if we increased the daily dose, the latent period could be reduced. If we gave 10 mg. per 100 gm. of the nucleoside by intravenous injection (that is, by one single intravenous injection), we could always induce nephrosis; and there was always a latent period which was a very well-defined one. Its duration was 5 days. All the other changes which we associate with the nephrotic syndrome appeared. The duration of this disease was much the same as that of the disease when it was induced by subcutaneous injections.

Once these animals recovered from their disease, we reinjected some of them within 2 days to one week after proteinuria had disappeared. We could then obtain a fresh nephrotic syndrome, as it were, with a slightly shorter latent period, but which did not spontaneously clear up, and which progressed to chronic renal failure, with subsequent death of the rat in a variable length of time from about 3 to 8 months after the onset of the second disease. This chronic disease, as we call it, has features very similar to the nephrotic syndrome. There is hyperlipemia, hypoproteinemia, gross proteinuria, urea retention, but not so much edema. Edema is present at the very beginning of the disease, then very rapidly disappears, and does not recur.

I should say that some of our rats followed for a considerable time once they have recovered from the acute disease, tend to develop proteinuria again after about 3 months. In 2 rats we followed for this length of time, proteinuria has returned to a minimal degree, with slight biochemical changes.

The latent period, which is always observed before proteinuria appears, made us wonder whether we were dealing with a substance which might be acting as an antigen, and that the rat, during this latent period, was producing an antibody to the substance which we were injecting. In order to test this hypothesis, we planned a few experiments. One was to do what Dr. Lange [3] did with his duck-serum disease; that is, give cortisone or ACTH and see whether we could prevent the onset of the disease by giving either cortisone or ACTH. Using fairly big doses of cortisone, we have been quite unable to prevent the onset of the aminonucleoside disease; and, similarly with ACTH, we have been unable to prevent the onset, or alter its course in any way.

Another experiment was to do what Kay [4] did to prevent duck-serum disease in rabbits; that is to irradiate our rats so that antibody production was inhibited. We have irradiated a number of these rats with a total body irradiation which is sufficient, according to other published work [5], to prevent the formation of antibodies to an antigen. Two days later, when there was complete inhibition of antibody production, we injected them with aminonucleoside. The disease in those rats which were given the substance intravenously appeared at exactly the same time as it did before radiation, and was accompanied by the same biochemical changes, and so on. The animals were usually

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[3] Wachstein, M. and Lange, K., Abstracts of 27th Annual Meeting of Society for Pediatric Research, p. 17, 1957.

[4] Kay, C. F., The mechanism by which experimental nephritis is produced in rabbits with nephrotoxic duck serum. J. Exper. Med., 72: 559, 1940.

[5] Fitch, F. W., Wissler, R. W., LaVia, M. and Barker, P., The timing of antigen injection relative to whole body X-irradiation and the development of circulating antibodies and the splenic histologic reaction in the rat. J. Immunol. 76: 151, 1956.

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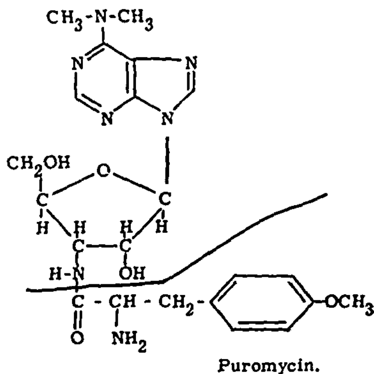
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6-dimethylamino-9-(3′-*p*-methoxy-L-phenyl-alanylamino-3′-deoxy-D-ribosyl)-purine

Fig. 78. Formula of Puromycin. Removal of the tyrosine molecule leaves a 3-amino-riboside of 6-dimethylaminopurine with which nephrosis is obtained.

DR. McCRORY: I can give some information in a rather different way, which might be of interest. Dr. Liu has succeeded in growing rat kidney in tissue cultures[6]. These rat kidney tissue cultures exhibit species-specific cytotoxic effects when rat anti-kidney sera is added. She has found that this aminonucleoside is cytotoxic in vitro to these cells. This is really a nucleoprotein antimetabolite; isn't it?

DR. WILSON: It could be; yes.

We were interested to know whether this disease could be produced in animals other than rats; so we have administered it to other laboratory animals (the rabbit, mouse, guinea pig, and dog). In none of them have we been able to find proteinuria or any of the biochemical changes which we associate with the nephrotic syndrome. This we thought was rather odd and strange; so we wondered whether there was some difference in the handling of this substance by these animals. This is more or less the point we have reached in studying this disease.

Figure 78 shows the formula of puromycin. If the tyrosine molecule is removed the nucleoside which has been used to induce nephrosis is obtained. It is a 3 amino riboside of 6-dimethylaminopurine.

We were also able to obtain from the Lederle Company the purine itself. It caused no proteinuria in rats. Others have succeeded in obtaining the amino ribose, and have also found that this does not cause proteinuria in the rat[7, 8, 9].

So we thought it was possible that the guinea pig and the other laboratory animals which did not get the disease might be able to break down the nucleoside into the free base and sugar, and so not get the disease, while the rat was not able to do this.

We set out to look for the aminonucleoside in the urine of rats and guinea pigs, and so on, hoping we might be able to find in the guinea pig and in the rabbit the free base and, in the rat, that we might find the aminonucleoside but no free base.

By using a Dowex 50 ion exchange column, and fractionation of the urine for purines and nucleosides, we have been able to show that the nucleoside appears in the urine of the rats and also of the rabbits and of the guinea pig.

DR. HEYMANN: In similar amounts?

DR. WILSON: Roughly similar amounts; yes.

From this, we infer that the nucleoside, if it is the nucleoside itself which is causing nephrosis in the rat, also is available to the kidney of the rabbit and of the guinea pig, and that the reason the guinea pig and the rabbit do not get the disease is not that they are breaking down this compound.

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[6] Liu, C., McCrory, W. W., and Flick, J. A., Cytotoxic Effects of Nephrotoxic Serum on Rat Tissue Culture, Proc. Soc. Exp. Biol. and Med. 95:331, June, 1957.
[7] Gidez, L., Personal communication, 1957.
[8] Stern, J., Personal communication, 1957.
[9] Recant, L., Personal communication, 1957.

We do not know what the reason is; there must be some other difference in the handling of this substance.

DR. METCOFF: May I make a couple of comments? The studies of Drs. Wilson and Heymann are an example of how nicely one can improve upon a preliminary observation. However, it may be of some interest that, very early in the game, some 2½ years ago, as I think I mentioned at the last conference, Dr. Joe Ferrabee was also interested in the question of antigen-antibody activity. He demonstrated quite effectively, he thought - I am not competent to judge - that the aminonucleoside had no hapten group and no antigenic activity.

At the same time, we treated a large group of rats with ACTH and cortisone, and had exactly the same experience Dr. Wilson has described; we were unable to prevent or modify the disease.

Neil Bricker[10] has produced renal disease in dogs, I am not sure whether it was the nephrotic syndrome, using the aminonucleoside. So presumably it is possible. Actually, he perfused an anoxic kidney with the nucleoside.

DR. HEYMANN: How many dogs did we have?

DR. WILSON: Just two.

DR. HEYMANN: With the same dose that rats were injected with?

DR. WILSON: Yes, the same dose.

DR. HEYMANN: Then it still remains true, that guinea pigs, rabbits, and mice do not develop the disease.

DR. METCOFF: Also, it seemed obvious that one ought to look for what might be an antimetabolite in this rat. Before embarking on that too extensively, I thought I would write to Lederle[11], because I imagined that they had explored every variety of this compound in relation to producing this disease, or preventing it. It turned out that they had explored or knew about a variety of the split products of the aminonucleoside but had not been able to prevent or produce the disease with any of these derivates.

DR. HEYMANN: In rats?

DR. METCOFF: I believe it was in rats.

DR. HEYMANN: We have a vew varieties which did produce the disease in rats.

DR. METCOFF: Which ones?

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[10] Bricker, N. S., Lubowitz, H., Dewey, R. R., Stokes, J. S., and Goldman, D. Y., The Functional Capacity of Persisting Nephrons in Experimentally Induced Chronic Bright's Disease. Proc. Central Soc. for Clin. Research 30: 15, 1957.

[11] Oleson, J. J., Personal communication.

DR. WILSON: The top half of Figure 78 shows the 6-dimethyl compound. We have tried 6-diethyl and 6-dipropyl compounds; and we have not seen any renal disease with these compounds. We have succeeded in producing the nephrotic syndrome with the 6-methyl propyl compound.

DR. HEYMANN: This was a very severe disease, a clear-cut one.

DR. WILSON: We have also tried the inosine form. It is completely benign, very much more benign than the others.

DR. RILEY: May I ask what the histological findings are on these rats which develop the clinical syndrome?

DR. WILSON: Dr. Donald B. Hackel has been studying these; and he finds some vacuoles in the tubules, but he also found basement membrane thickening.

DR. HEYMANN: Dr. Hackel told me that the histological alterations are the same as the ones seen in the disease produced by nephrotoxic sera. The only difference being that in early phases, the tubular changes seem to be more marked in the aminonucleoside disease.

CHAIRMAN BARNETT: Dr. Spater has been studying this disease a little, too.

DR. SPATER: We have used aminonucleoside in a few animals and produced edema 6 days after a single intravenous injection. Yet despite the obvious edema these few animals lacked evidence of basement membrane thickening.

DR. GOODMAN: Are their tubules knocked out?

DR. SPATER: I would like to show some slides of the very early stages later in this meeting.

DR. GOODMAN: If you are able to find an immune mechanism, it would be most interesting. However, much of this sounds somewhat familiar to me, in terms of another substance which is also cytotoxic. When one injects uranium intravenously in high enough doses in a rat, a nice proteinuria occurs quite early; but, if one injects much smaller doses, about a tenth of a milligram of uranium into 150 gram rats, nothing happens for two or three days. Then proteinuria begins. Why the onset of proteinuria is delayed I have no idea. We thought perhaps it took time for this nephrotoxic agent to localize so it could do enough damage. I would like to ask whether you can shorten the length of the latent period if you give a large enough dose of aminonucleoside?

DR. WILSON. You kill the animal; but you do not --

DR. HEYMANN. That is right. The uranium effect may not represent a true latent period. It may be something that, with a small dose, starts immediately but slowly, and for the first few days it may remain imperceptible.

DR. GOODMAN: I am just asking if the aminonucleoside effect might not be analogous to that produced by uranium.

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DR. GOODMAN: I am just asking if the aminonucleoside effect might not be analogous to that produced by uranium.

DR. HEYMANN: You just cannot get rid of the latent period with the aminonucleoside.

DR. JAMES: There is just one small contribution I can perhaps make to the aminonucleoside story. We studied one dog only, but after about three weeks, this animal did develop very significant proteinuria, with some decrease in serum proteins. We had planned to continue this study, but unfortunately we ran out of aminonucleoside. The animal subsequently recovered and lost his proteinuria. There is no question, I think, that this animal did develop clinical evidence at least, of renal damage following these injections.

I know that Dr. Gidez at Brookhaven has also been interested in compounds related to aminonucleoside, but none of the substances he has tested has produced comparable hyperlipemia in rats.

DR. HEYMANN: Did you inject your dogs daily for three weeks by subcutaneous injection?

DR. JAMES: For at least three weeks or longer. I am sorry I do not have more information about these studies at present. I would imagine that a dosage phenomenon is involved, and that a nephrotic syndrome might well be produced in dogs if you gave the appropriate dose.

DR. HEYMANN: We injected dogs for as long as 30 days.

DR. JAMES: For obvious reasons, it would be useful to have a much larger experimental animal.

DR. METCOFF: I would like to suggest that this be tried in puppies.

CHAIRMAN BARNETT: Since the histology of the kidney has come up, perhaps, Dr. Spater, you could show the material you have obtained in this disease.

D. Dr. H. W. Spater

DR. SPATER: Last spring we presented data which showed, in animals which had been injected with a weak nephrotoxic serum, a transient proteinuria and a very mild disease. The kidneys of these animals demonstrated a diminution of alkaline phosphatase activity in the juxtacapsular area of the cortex. In addition, the proximal tubular cells also showed a decrease in acid phosphatase-rich droplets normally found here.

Similar methods were used on the kidney of aminonucleoside treated rats. I shall describe some of the essential features of the histologic sections.

A section from the kidney of an animal killed two days after an intravenous injection of aminonucleoside appears normal and there is no demonstrable lipid in the tubular cells. We find that lipid accumulation is an early indicator of cell abnormality.

If the animal is sacrificed six days after the intravenous injection there is a characteristic accumulation of lipid droplets in the basal portion of the proximal tubular cells.

Not all proximal tubules are involved. The lipid droplets appear to be segmentally distributed along the proximal tubule.

We thought that the lipid would be a very convenient way to tag cells which we believe to be abnormal and then superimpose our enzyme techniques to reveal any relationship between changes in enzyme activity in these cells and the appearance of lipid.

This relationship apparently exists since the lipid-containing tubules show considerably less alkaline phosphatase activity than the non-lipid containing tubular segments.

DR. HEYMANN: How many days after the injection were your preparations obtained?

DR. SPATER: The rats were killed six days after the injection.

Even in the cells which have diminished alkaline-phosphatase activity the brush borders are still there; by H and E stain, all these cells look perfectly normal.

DR. METCOFF: Isn't that a function, Dr. Spater, of where you take your cross-section? I mean I could see areas in your sections along the longitudinal length of the tubule where, if one took a cross-section, one would get a very dense stain in the luminal area, and no droplets, for example, about two-thirds of the way down.

DR. SPATER: That may be true in this particular section shown here, however, the finding appears quite consistent when examining a large number of sections.

DR. METCOFF: That is not the same tubule, is it, with different undulations which are sectioned?

DR. SPATER: I suspect that this may indeed be the case and suggests a spotty distribution in the proximal tubule. Of further interest is the fact that the enzyme activity is not completely gone since the enzymatic hydrolysis period was extremely short, on the order of five minutes, so that only the initial activity of the enzyme is visualized. Further incubation, let us say fifteen minutes, produces sufficient product of reaction to make all the tubules appear to have similar activity. One could easily miss the difference in activity with prolonged incubation.

I would like to mention briefly some of the questions which have arisen in studying the normal kidney by showing a section of normal rat kidney stained for acid phosphatase. The proximal tubules show the characteristic acid phosphatase-rich droplets in the cytoplasm.

DR. KRETCHMER: How old were the rats?

DR. SPATER: These were young rats; they were about 45 to 60 days old. They weighed about 70 to 80 grams.

DR. KRETCHMER: Then these rats do not have the usual proteinuria.

DR. SPATER: That is correct. We also have noted that the PAS positive droplets appear to be identical to the acid phosphatase droplets by their appearance in the normal cell and the increase in their size and number following egg white injection.

This has raised several perplexing questions as to which direction we should lean in trying to interpret what these PAS droplets are. Are they what Dr. Jean Oliver says, mitochondria coated, or associated, with reabsorbed protein from the glomerular filtrate? If they are, they should not be acid phosphatase-rich, because acid phosphatase is not characteristically a component of mitochondria.

On the other hand, are these droplets what Dr. Werner Strauss suggests they might be? In one of his droplet fractions from kidney, he has isolated a fraction similar in size to these droplets, which are both PAS (positive) and acid phosphatase-rich.

I would welcome a comment from Dr. Kretchmer on this question.

DR. KRETCHMER: I always find myself in the position of a droplet defender. (Laughter)

There are a few questions which come to mind, which I think Dr. Spater could answer. One is, did you say these droplets disappear with proteinuria?

DR. SPATER: We have previously demonstrated them to be decreased in number and activity in the antibody induced disease.

DR. KRETCHMER: Dr. Oliver does not say that droplets are mitochondria associated with egg white. I think what he actually showed, as did Rodin with the electron microscope, was that mitochondria dissolve or disintegrate, and that maybe pieces of the mitochondria coalesce to form the droplets.

Then Strauss showed that these droplets contained relatively large amounts of egg white measured immunochemically. He truly took non-random groups of droplets, since he isloated the droplets with cellulose, and ended up with about one per cent of what was the predicted total droplet population.

What Oliver maintains, as you know, and what was brought out last year, is that droplet formation is a normal phenomenon, it occurs during proteinuria, and is much more marked when there is a foreign protein. In his dissections of human kidney obtained from soldiers who had acute hemorrhagic fever, and had received large doses of "salt-poor albumin," he showed droplet formation. They were gram-positive and PAS positive.

DR. SPATER: *How did you demonstrate the presence of acid phosphatase?*

DR. KRETCHMER: I never did.

DR. SPATER: This is a lead phosphate precipitate.

DR. KRETCHMER: Then that makes it even simpler. You are defining an acid phosphatase as something which will hydrolyze a phosphate at an acid pH.

I do not know if mitochondria will convert organic phosphates into inorganic at an acid pH. I think it might be worthwhile to fractionate and see if you can do that in vitro.

The other thing I do not quite understand is how you reconcile your electron microscope observations with Rodin's findings using egg white. I think he clearly showed that droplets formed after egg white administration.

DR. SPATER: The limited electron microscope studies we did were on normal kidney in which PAS positive droplets were already present.

DR. KRETCHMER: Yes; in Oliver's paper, he says this is so.

DR. SPATER: Yes; but you cannot see anything which resembles these droplets; you just see normal, rod-shaped mitochondria.

CHAIRMAN BARNETT: I think maybe we had better leave this for an after-twelve o'clock continuation.

I would like to ask Dr. Meyer if he could spend a few minutes telling us some of the things they are doing.

E. Dr. Reuben Meyer

DR. MEYER (Detroit, Michigan): Mr. M. D. Poulik in our laboratories has collaborated with Dr. Wolf W. Zuelzer and I on studies of the protein changes in sera and urines of nephrotic children. We are using the filter paper as well as the starch gel electrophoretic methods. I presume most of you are familiar with the starch gel technique[12].

Up to this time we have studied about 15 to 20 patients with these methods. On paper the patterns corroborate findings of previous workers. Since the alpha-2 globulins is found consistently elevated and since starch gel electrophoresis is able to resolve this region into 7-9 discrete protein entities, some of them being genetically determined, our attention was focused to this region. We have found one protein, the so-called "slow alpha-2 globulin," to be consistently elevated in our patients. This protein is a sugar protein of high molecular weight and was isolated in crystalline form by Dr. R. K. Brown[13]. In urine this protein and some of the haptoglobins were never found in our patients.

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[12] Smithies, O., Biochem. J. 61: 629, 1955.
[13] Brown, R. K. et al., J. A. Chem. Soc. 76: 4244, 1954.

At the present time we are engaged in defining the role of this protein and its distribution in tissues by the fluorescent antibody technique. During these studies, which also include some myeloma patients, we found a protein in a case of myeloma which has the same electrophoretic mobility as the slow alpha-2 globulin. We are studying its immunologic relationship with the slow alpha-2 in the nephrotics. These studies are preliminary and form a base line for our tissue studies.

I have no slides, but I do have some photographs, if anybody is interested.

We are also beginning to study the distribution of the proteins by two dimensional starch gel electrophoresis [14] which gives an even better insight into the presence or absence of the various protein components since this method was able to resolve normal human serum into 21 separate protein fractions.

CHAIRMAN BARNETT: I think as always happens to us, we are going to run very short of time during the last day. We have not heard from such well-known stars as McCrory and Kretchmer; we also, unfortunately, have not heard from a couple of the people who have not been with us in the past, and who are doing some very interesting work, Dr. Boggs from Chicago, and Dr. Katz from Cedars of Lebanon.

I think, in the few minutes left, we would like to hear, Dr. Katz, what you and Dr. Sellers are doing; then we will close.

F. Dr. Joseph Katz

DR. KATZ (Los Angeles, California): I will manage in three minutes.

Our group at the Institute for Medical Research of the Cedars of Lebanon Hospital (Dr. Alvin L. Sellers, Dr. Sheldon Rosenfeld and myself) has tried for some time to develop an isolated perfused kidney for metabolic studies. It is obvious that you can do a great deal with such a preparation. However, the kidney offers a great deal more difficulty than perfused liver. So far nobody has succeeded in perfusing a functioning kidney in vitro with whole blood. In previous preparations it was always necessary to introduce a lung into the circuit; otherwise there was immediate vasoconstriction, and the blood flow to the kidney stopped. In classical physiology it was said that the lung had to "detoxify" the blood.

Our preparation is similar to that used by Miller for liver perfusion. The system consists mainly of a pump that circulates the blood, an oxygenator, and the rabbit kidney - all enclosed in an air-conditioned box. Blood for the perfusion is used undiluted, and is taken from rabbits by heart puncture without anesthesia. The kidney is removed under alcohol anesthesia. This may be a significant point. The blood flow through the kidney is about 20 ml. and in our best preparation about 30 ml. per minute.

DR. GOODMAN: Per minute?

DR. KATZ: Yes, 15 to 20 is common. However, the preparation so far has been highly variable. Thus sometimes we had no urine flow at all, and sometimes diuresis.

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[14] Poulik, M. D. and Smithies, O., Biochem. J. (to appear shortly).

In some cases we had one ml. of urine in 15-20 minutes, in others one ml. every 2-3 minutes.

Blood and urine flow per se do not indicate whether the kidney is physiologically functioning. We have used para-amino hippuric acid clearance as our criterion of function. In our very best preparation we obtained an 80 per cent clearance (as measured by AV difference). Usually we do not do as well, and get about 50 per cent clearance. We also observed some good creatinine clearance; also in our best experiments there was little glucose in the urine. One of our difficulties is that our preparations are quite erratic, and often we get no clearances at all. The preparations break down within two or three hours. We do not know yet what is lacking in our system to cause this breakdown.

The other criterion for function we have used is the response to hormones, namely renin and pitressin. With renin there was increase in urine flow and proteinuria. When the urine was examined electrophoretically the changes were similar to those seen with renin treated animals. We have also had a response to pitressin with a diminished urine flow.

CHAIRMAN BARNETT: Thank you.

DR. JANEWAY: That is a lot of work in a few words. (Laughter, applause)

CHAIRMAN BARNETT: *That was very quickly said. We have two minutes left* for questions. (Laughter)

Next, I thought we might turn to some of the clinical aspects in terms of treatment and clinical investigation.

It seemed to me it would be useful, in discussing treatment, if we addressed ourselves to rather specific questions, and asked the group if they have data related to these specific questions.

It seems to me our present situation in regard to treating children is such that what we really need to know is whether anyone has any evidence, which is not known to the rest of us, of the superiority of any one form of treatment over another.

DR. LANGE. What is the base line? (Laughter)

CHAIRMAN BARNETT. Granted that the answer to this question is "no" (laughter) then I would ask if anyone has set up any systematic clinical investigation using a valid control series by which they are attempting to answer this question.

I have introduced the problem in this way to try to orient the discussion along lines which either report or could lead to a systematic investigation of what I consider to be the important thing, which is not what our individual experiences have been during the last year, but how can we answer the two most important questions: First, how is the administration of steroids affecting *this disease, both symptomatically* and, more importantly, in terms of the ultimate outcome, secondly, if they are, or if we think they are, what is the best way for us to be using steroids?

I might say, to begin with, that I have been amazed, in the years we have been having these conferences, to see new clinics set up which are just starting to treat large groups of children, and to hear, during the first year or two of a new clinic, what to me were always rather amazing successes in the treatment of these children; whereas, in clinics which have been following children for longer periods of time, the results were never as good.

I have had the experience, in the last few years, of going from a clinic where we have been seeing children for a long time, and where my view of the situation was a little less optimistic than that of others, to a new clinic where we are starting to see or gather a new group of children.

I could report, on the basis of our experience at our new hospital during the last two years, that our successes are practically 100 per cent.

DR. LANGE: Maybe your treatment is better. (Laughter)

CHAIRMAN BARNETT: However, I occasionally go down and see Dr. Kretchmer at the New York Hospital. (Laughter)

In patients who are treated essentially in the same way, I realize that our new clinic is young and that this is an important factor.

I am also interested in variations of optimism among various people from year to year in this disease. I think perhaps, apart from individual experiences such as Dr. Lange's and those of a few others among us, the major effort which has been made thus far in attempting a statistical evaluation of the effects of steroid treatment has been that which came out of the discussion during our meeting in Cleveland several years ago and was done by Dr. Riley. We might start by asking Dr. Riley to tell us what his present thoughts are on this subject of evaluation of therapy.

V. Evaluations of Steroid Therapy

A. Dr. Conrad M. Riley

DR. RILEY (Babies' Hospital, Columbia Medical Center, New York): I would like to talk about evaluating my own cases as a model for what I hope will be another cooperative study.

I remember that it was quite a lot of work for all of you, three years ago, to get your data together, and I think I can simplify it this time a great deal.

Figure 79 is what I have been able to do with my own group (A) compared with the pooled group (B). You remember the previous evaluation[15] was at the end of only 30 months, 2½ years after onset of edema. We have been able to carry this present comparison out to 3½ years. But, by the end of this time, because so many of the cases

[15] Riley, C. M., Davis, R. A., Fertig, J. W., and Berger, A.P., Nephrosis of childhood: Statistical evaluation of the effect of adrenocortical-active therapy, J. Chron. Dis., 3: 640-650, June, 1956.

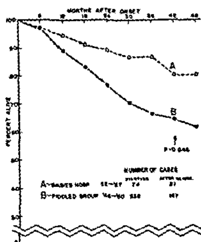


Fig 79 Survival curve of recent Babies' Hospital group of patients (A) compared with earlier groups (B)

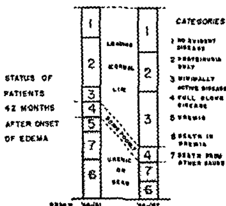


Fig. 80. Comparison of status of patients with onsetof edema in the pre-steroid days with that of current patients at a period 42 months after onset of edema. Each bar represents 100% The earlier group is comprised of 51 cases, the latter group of 26 cases.

occurred recently, my own group has dropped to only 21 cases, and one is on pretty shaky ground in a statistical evaluation based on 21 patients. When I tried to make a comparison of my own more recent group against my earlier group, the numbers involved were not sufficient to bring out any statistically significant difference. I think we got a P of something in the neighborhood of 10 per cent, which is not very good.

So then I turned to the previous, combined group I had collected from all you people, and used it for comparison purposes. Line B is this pooled group where disease began, 1946, to 1950 in the figure. Using my group (line A) against the combined group (B), I get a fairly respectable P, of less than 0.05.

On the other hand, Dr. Fertig, our statistician, says "It is not fair for you to carry your own group against everybody else's; maybe there is something different about the way you personally are doing things. Therefore, I would not put too much faith in such a comparison."

This led us back to the problem which Henry brought up, would enough of you be willing to send in enough data on all patients you have seen with an onset of edema since January 1, 1952 and let us make up a comparable table?

The only thing which gives me a little bit of confidence in this comparison is that my own small group, which does not have the same statistical significance as the larger 1946 to 1950 group, practically superimposes itself on the over-all experience, so that I think the difference between my methods of handling patients and those others was not enough to account for the discrepancy in survival.

But it should be much more convincing if we had a pooled group of those with an onset since 1952; I think we would probably be able to get significant differences at least to 48 or 52 months after onset, if we did that. If there is agreement I would like to collect the data and present the analysis to you.

The other question Dr. Barnett raised is, "What is the status of the patients who have survived in addition to the difference in the percentage of those who have died?"

In Figure 80 we made a crude attempt to evaluate this on our own patients. Again, we are limited by the numbers of patients available to us.

In the group with onset of edema between 1946 and 1951 the total 100 per cent represents 51 patients, and in the more recent group, onset 1952 to 1957, it represents only 26 patients.

So I am talking in terms of fairly small groups, and I would not attach any statistical significance to this.

Our technique, in brief, was, at each six-month interval after the onset of the disease, to evaluate a patient's status based on the previous six months' performance. The categories selected are shown in the figure. 1) absolutely well; 2) well except for proteinuria; 3) perhaps a little bit of edema, or at least enough signs with proteinuria so that we thought treatment was necessary; 4) full-blown, crippling disease; 5) having passed beyond the point of no return into either 6) death from uremia, or 7) from another cause.

It is interesting that, as you compare the two groups, the major difference is obviously between the groups which are past the point of no return. The main difference among the living appears to be made up in group 3 of children who still are showing signs of some active disease, whereas the difference in percentage between those whose disease has subsided (groups 1 and 2) is not very great.

So it looks as if the contribution has been to keep the disease from progressing to death or uremia in this very small group.

To make this kind of analysis on a pooled group would require a tremendous amount of work on the part of a tremendous number of people; and I doubt if we would ever get the answers back.

I think if we could make the more simple comparison of just living and dead, we could probably get the answers back very quickly, so I make no plea for this sort of detailed analysis on a large scale - I would like simply to evaluate those dead and alive, depending on the duration of their illness.

DR. GRIBETZ: Isn't yours a somewhat biased series? As you become more and more involved with this disease, aren't you getting the "tougher" cases referred?

DR. RILEY: The way I am trying to get around that, Don, is by including in these groups only those we have seen within the first six months of their illness. If I had a large enough group, I would like to include only those I saw within the first three months, so those who are referred to me after a year or eighteen months, and are doing poorly, do not get considered in this.

DR. HEYMANN: When you get the data from all of us, don't you think it would be a good idea to have one group starting in 1954 separately?

DR. RILEY: This limits us on the length of time we can carry out the analyses.

DR. HEYMANN: That is correct, but from 1952 to 1954 many of us still were satisfied with short-term treatments for edema only. From 1954 on, most of us adopted a more vigorous form of treatment.

DR. RILEY: In my own group, the reason I chose 1952 was that about that time we started to use more aggressive treatment.

DR. HEYMANN But many of us did not.

DR. RILEY: If we get anything interesting out of analyses of patients from 1952, we can break it down to 1953 or 1954.

DR. HEYMANN: Or, if you could separate children treated with long-term intensive treatment from onset, from those that were not treated right from onset with intensive courses of steroid administration, there may be an impressive difference.

DR. RILEY The difficulty with that is, Dr. Heymann, that there is a lot of selection as to who gets the long-term treatment. For example, the patient who does not

tolerate large doses of steroids, gets thrown out of that group; and the comparison then becomes weighted by dropping out patients who are doing very poorly, and who never get put onto a maintenance schedule, because they cannot be.

That is why we elected to do it by time although, obviously, there is always the question, has the disease changed in character? It looked as if it were the best comparison we could make.

CHAIRMAN BARNETT: I think perhaps we should discuss whether we should try to receive data from people. It might be worthwhile to look at Dr. Kohn's long-term data, which might help us to decide how this might be approached.

DR. METCOFF: May I make a comment at this point? What Dr. Riley has tried to do seems to me extremely useful and important.

However, we must not feel too secure about the method of handling the data. I have been very concerned about this; yet every biometrician I have talked to seems satisfied with this approach.

I have, however, sought assistance from one of the mathematicians at the University of Chicago, who is interested in the handling of biological data. He also was dissatisfied with this technique of handling the material. He was not convinced that it provided an appropriate mathematical analysis of what is happening to the course of the disease. He is now taking this under consideration regarding a better method of approach. My own knowledge is so limited that I cannot offer one, at present.

I think we should not be completely complacent about the technique of handing the data, although it appears to be better than anything anyone of us has used in the past.

DR. RILEY: I can only say that I have sought the best advice we have, and have worked it out with Dr. Fertig; I am not qualified to do anything but quote him.

B. Dr. Jerome L. Kohn

DR. KOHN (Mt. Sinai Hospital, New York): May I show some of our data, since our time is short?

I have been following patients with the nephrotic syndrome for as long as 32 years. At present Dr. Donald Gribetz is associated with me in this study.

The patients have been divided into three groups. We have attempted to compare the morbidity and mortality of each group and note the effects of the a) antibiotics or sulfonamides, b) steroid plus antibiotics and c) to compare the use of these agents with the natural history of the disease prior to the use of these agents. These patients have been followed in our kidney clinic which has been functioning for about 30 years.

Table 40 shows the mortality in each group. In the pre-antibiotics group of 33 children 63 per cent are known to have died. In the antibiotic group of 24 again 63 per cent are known to have died. We can conclude therefore that antibiotics did not alter

ERRATA

Caption, Fig. 4, page 8, line 5 - remains (not rains)

Caption, Fig. 15, page 29, line 4 - immediate (not immedeate)

Caption, Fig. 35, page 77, line 2 - slide (not slice)

Caption, Fig. 56, page 140, line 2 - absence (not abscence)

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TABLE 42

NEPHROSIS: DATA OF LIVING PATIENTS

	Total No. Pts.	Known Living	Seen Recently	Duration of Follow-up (Years)	Edema 0 Albuminuria 0	Edema 0 Albuminuria +	Chronic Nephritis
I	33	11	6	16-32	1	4	1
II	24	8	8	10-14	3	4	1
III	24	21	21	0.6-7.3	8	9	4

Table 42 shows a follow-up of the living patients. In group I, 6 of 11 have been seen by us recently of whom one has a moderate nitrogen retention. All of the patients in group I are clinically well and living a normal life. Four still have albuminuria. Several have gone through normal pregnancies.

In group II all 8 have been seen recently. Seven are clinically well and one has a moderate chronic nephritis.

In group III, 17 of 21 are clinically well. Of these 17, 4 still have an increased serum cholesterol and a decreased total serum protein.

About 50 per cent of the patients in all three groups still have albuminuria. We feel that if a child shows progressive improvement, has normal blood chemistry and normal blood pressure two years after onset, the chances of long survival are excellent. The presence of a moderate albuminuria does not seem to be an important factor.

DR. GRIBETZ: Since, as Dr. Barnett has pointed out, nobody seems to be willing to conduct some type of alternate study, the only way to assess the value of steroids is to compare the results obtained with those achieved in the years prior to their use. We think this is the chief value of our data.

One of the striking things which appears from these data is that in both groups I and II (group I being the one in which neither steroids nor antibiotics were used and group II being the one treated with antibiotics but no steroids) the mortality at the end of four years was 66 per cent. The figure was exactly the same in the two groups for some poorly understood reason. Nevertheless, perhaps four years' survival after the onset of the disease is crucial.

In our present group, treated with both antibiotics and steroids, even if we include three or four more children, who may succumb within four years after the onset of their disease, the mortality figure will be about one-half that of the previous experiences.

DR. KOHN: We must ask ourselves the following questions: are the steroids

a) good diuretic agents?
b) do they influence morbidity?
c) do they influence the eventual outcome?

We can say that thus far steroids are the best known therapeutic agent. The length of time with edema in the first two years has been halved. We believe our mainenance therapy after discharge from the hospital has been a factor. The question of eventual outcome is still speculative. It appears from our accumulated data that steroids may influence the prognosis favorably.

DR. GRIBETZ: Whether or not we are just delaying the time of death, whether these children will die within 8 years instead of within 4, is difficult to say at the present time. We do think, however, that groups I and II may serve as a basis for comparison of the forms of therapy currently in use.

CHAIRMAN BARNETT: Would it not be fair to say at present, that in relation to all the other forms of therapy which have been suggested or to any which are presently available, therapy with the adrenal steroids appears to be the most useful? There can be no question that the children are symptomatically better with the various forms of steroid therapy we are using; and there are instances with some fairly good supporting evidence that, at least during the first four years of the disease, survival is increased.

The question of what the ultimate outcome is going to be is really something we cannot answer at the present time; and it seems to me that perhaps we should accept these two statements I have made. What we really can look into at present, and need to, is what is the best of the many regimens which are being used to achieve the best possible results with the steroids.

I think this has to be done in a systematic fashion. Without spending a great deal of time (because I am not sure it is worth a great deal of time), if some of us were willing to undertake a program of treatment wherein we have our own controls - and these are not treated versus untreated, because I think we would not do that - but between two, or possibly three, different regimens of treatment. If we were to select children randomly and in a consistent fashion, we might gain useful information within the course of the next two, three or four years. I do not think this would involve very much work other than analysis of the data such as we are now doing.

I do not think we have time today to conclude that we should do this, or to make the many decisions which would be necessary if any of us were going to join in such effort.

I think, however, it would be worthwhile, if we agree that this is worth considering, to hear from one or two people who represent sort of opposite ends of the spectrum of how treatment is being given, and learn what their regimen is at present.

I think perhaps Dr. McCrory and Dr. Rapoport represent one group who, in a way, use minimum steroid treatment, that is, of relatively short duration. They have a fairly well defined plan of approach. I think Dr. Lange, on the other hand, also has a well-defined plan which he has been following for many years.

I thought, if we could hear from these two groups briefly, and in addition, think of what might be done in the way of a controlled, collaborative study, this session will prove to be useful.

I think, as part of this, Dr. Kramer, in collaboration with Dr. Kretchmer and others, has some data on a few patients treated with a new drug, with which most of us have not had experience; and it would be worth hearing about this.

DR. METCOFF: I might also add that, whenever an individual has an opportunity to participate in the organization of a new clinic (for example, as Henry and I are now doing), this provides an unusual opportunity to set up a series which would not have to be biased at its outset.

CHAIRMAN BARNETT: I thought we might start with Dr. Lange.

C. Dr. Kurt Lange

DR. LANGE: I think our data will represent the extreme on one end, insofar as the same therapeutic regimen was carried out since 1951 on all children and adults with nephrosis coming under our observation; also they will probably be the extreme as far as dosage is concerned. Maybe this is the reason why we have the extreme in favorable results. (Laughter)

I want to stress that our clinic which treats nephritic and nephrotic children has existed since 1951. A good many patients were treated before that, in a sort of cooperative study, so that, actually, since steroid therapy with maintenance was first suggested by us, these children were all treated more or less by the same group.

The treatment consists of the following:

Every child or adult - and I want to stress that two-thirds of this group are children; one-third are adults above the age of 13 - when admitted, received a quick but thorough work-up. We try to make this period as short as possible for we feel that, the later treatment is started, the less effective it is going to be.

DR. RAPOPORT: This is just a feeling; this you can only tell after --

DR. LANGE: We have published the supportive data for this opinion[16].

DR. RAPOPORT: How long would you say early and late is? The difference between treating a child having the disease one month or three months?

DR. LANGE: We have arbitrarily set, in these statistics which we have published in the Archives of Internal Medicine, I think, three months as the dividing line, so that a few days more of admitting work-up do not matter. I only want to stress that one should not sit and wait for a spontaneous remission.

DR. RAPOPORT: This is an area with a question mark over it, you mean the first diuresis after the onset?

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[16] Lange, K., Strang, R., Wenk, E.J., Slobody, L. B., The Treatment of the Nephrotic Syndrome with Steroids in Children and Adults. Arch. of Int. Med. 99: 760, 1957.

DR. LANGE: That is right; from the beginning.

All of them were then treated in the following way: If their weight was under 40 pounds, they received 120 units of aqueous ACTH, divided in 4 doses; that means 30 units q.6.h. around the clock for a minimum of 12 days. In the past two years, if we could get away with it, we extended the initial treatment to 21 days, according to the suggestion of Danowski and his group. Children over 40 pounds, up to the weight of 80 pounds, received 160 units of ACTH a day; and everybody over 80 pounds received 200 units of ACTH a day, divided into 4 doses.

Provided diuresis has occurred, but only when diuresis has occurred, maintenance therapy is started. This maintenance therapy consists of 300 mg. of cortisone a day, divided into 3 doses or, when 400 mg. are given, into 4 doses - that means 100 mg. per dose, for 3 successive days out of each week, followed by 4 days of rest, then again the same cycle. Children under 40 pounds of weight received 300 mg. of cortisone per day, all others 400 mg. per day.

This is continued for a minimum period of one year; and, in cases which started out with a rather severe nephritic background we have continued maintenance up to 18 months.

DR. HEYMANN: Even if their urine and chemical values became normal?

DR. LANGE: Even if they became normal; and many of them became normal, as far as all biochemical values were concerned, none of them was exempted from the scheme, except when they got chicken pox. Under this circumstance therapy was interrupted. This was the only reason for interruption; whether this reason is right or wrong I do not even know.

DR. METCOFF: What about the other infections, Kurt?

DR. LANGE: We have not interrupted the treatment in the presence of other infections. In some infections, when they were very severe, we have even increased the doses of cortisone, but covered them heavily with antibiotics. Table 43 represents a statistic which is made up according to the suggestion of Conrad Riley; we have now one great difficulty, however. We do not have controls beyond 65 months; for the period for the controls extends from 1946 to 1951, when all our cases were started on maintenance therapy. We do not have controls beyond that time. So we leaned over backwards; we assumed that, in the control group, none of the children would have died after this date. Then we compared it to the group treated with the therapy we mentioned.

With Dr. Riley's permission, we have used his figures in the control group, in order to make it larger; but I want to stress that our figures for the control group coincide as far as death is concerned with those of the total group and with Dr. Riley's. For statistical purposes, we have used the combined figures, i.e., our control group plus the larger control group of Dr. Riley.

There are 185 cases in the control group, and 46 cases in the maintenance group. The maintenance group comprises only cases who were under our type of therapy for

TABLE 43
THERAPEUTIC RESULTS IN PATIENTS WITH THE NEPHROTIC SYNDROME

Pooled data from group with "No Steroid Therapy" and "Steroid Therapy when Edematous" compared to group on "Prolonged Intermittent Steroid Therapy."

	CONTROLS									MAINTENANCE				
	PATIENTS									PATIENTS				
Period (mos.)	At Start		Entered		Died		Withdrew		Death Rate%	At Start	Entered	Died	Withdrew	Deaths Expected
	48				1		9							
0-5	112*	160			2	3	0	9	1.9		24	0	0	0.46
	38		0		4•		4							
6-11	110	148	14	14	7	9	26	30	6.4	24	3	0	3	1.54
	30		0		4		2							
12-17	93	123	4	4	6	10	10	12	8.4	24	6	0	5	2.05
	24		0		0		3							
18-23	81	105	2	2	6	6	9	12	6.0	25	3	0	4	1.47
	21		0		2		1							
24-29	68	89	2	2	6	8	7	8	9.3	24	1	0	2	2.19
	18		0		0		3							
30-35	57	75	2	2	6	9	5	8	12.6	23	3	0	1	3.02
	15		0		1		1							
36-41	48	63	1	1	2	3	6	7	5.0	25	1	0	5	1.13
	13		0		0		1							
42-47	41	54	0	0	1	1	4	5	1.9	21	0	0	3	0.37
	12		0		0		3							
48-53	36	48	0	0	1	1	2	5	2.2	18	0	0	5	0.34
	9		0		0		0							
54-59	33	42	0	0	0	0	7	7	0.0	13	0	1	4	0.00
	9		0		0		2							
60-65	26	35	0	0	1	1	5	7	3.2	8	1	0	2	0.24
66-71										7	1	0	1	
72-77										7	1	0	5	
78-83										3	0	0	1	
84-89										2	1	0	0	
90-95										3	0	0	0	
96-101										3	1	0	2	
102-107										2	1	0	0	
108-113										3	0	0	0	
114-119										3	0	0	0	
120-125										3	0	0	1	
126-131										2	0	0	0	
132-137										2	0	0	1	
138-143										1	0	0	0	
144-149										1	0	0	1	
TOTALS				185	51+						46	1		12.81

*Lower figures in controls from. Riley, C., Nephrosis Conference 1954.

$\chi^2 < 0.001$

$$\text{Death rate} = \frac{\text{Died}}{\begin{matrix}\text{Number at} \\ \text{start of} \\ \text{period}\end{matrix} + \frac{1}{2}\begin{pmatrix}\text{Number} \\ \text{entering during} \\ \text{interval}\end{pmatrix} - \frac{1}{2}\begin{pmatrix}\text{Number} \\ \text{withdrawing} \\ \text{during interval}\end{pmatrix}}$$

at least two years. An additional group, not under therapy for that length of time, is not included in these statistics for reasons which are quite obvious, but in this group too, no death has occurred so far.

We come then to an expected death rate for the group of 46 cases on maintenance therapy based on the death rate of the control group, *which would be 12.81 deaths.*

The actual death rate is 1.0. I think one additional case is going to die in the near future. *This is an adult with nephrosis subsequent to poison ivy, and a relapse after a* second exposure to poison ivy. This case is included, so the figure for the actual deaths may rise to two.

DR. RAPOPORT: He is still being treated?

DR. LANGE: *He is not being treated any more. He* has no edema, but his urea clearance is 7 ml/min.

We are left then with an expected death rate of 12.8, and an actual death rate of one, which gives us a chi-square value of .001.

CHAIRMAN BARNETT. Let me ask you. Of the 46 patients assessed, were these the total number of patients you have seen in this period?

DR. LANGE: Yes. The total number of cases, except those patients who have not yet been followed for two years after the onset of the disease.

CHAIRMAN BARNETT. I am wondering about patients who may have started with you, then have gone to another doctor.

DR. LANGE: No - we have none in this group whom we were not able to follow.

DR. GOODMAN: Along that same line, you said that those who diurese you then put on this regimen. You mean then that every patient you have started has diuresed and gone on this regimen?

DR. LANGE: *Yes, but we will come to that in a minute, in detail.*

All cases were followed. They were seen or at least heard of by letter from the parents within the last six months. They were non-edematous, and a urine specimen was obtained.

DR. GRIBETZ: You say your expected death rate would be 12 out of 48.

DR. LANGE: 12 out of 46.

DR. GRIBETZ. That is 25 per cent.

DR. LANGE: Yes, approximately.

DR. GRIBETZ: That is contrary to our data and to all other statistics.

CHAIRMAN BARNETT: Dr. Lange is using Riley's data for treated patients, which is close to your 33 per cent.

DR. RILEY: But also remember he has figures for the expected deaths until 60 months after onset.

DR. LANGE: We have no comparison beyond 65 months. We could use your figures, Dr. Gribetz, to fill in this gap.

DR. GRIBETZ: That is on expected death rate of untreated cases?

DR. LANGE: If they were not treated, you would expect, on the basis of the control group, 12.8 deaths.

DR. GRIBETZ: *Henry, on untreated cases, our expected death rate was 66 per cent.*

DR. JANEWAY: These patients were treated; but they just were not treated after the initial diuresis.

Steroids were used in many of them.

DR. LANGE: They were treated with antibiotics also during the initial course of steroid therapy and in many instances later received antibiotics in a scheme similar to the one used for the prophylaxis of rheumatic fever.

DR. RILEY: May I point out that you have included cases who have had the disease from 6 months to as much as 108 months, at which time they already may have survived a crucial time, and were going to get well. Also, by this technique you have failed to get those who died early in the course of the disease.

DR. LANGE: Yes. But all cases had full-blown signs of nephrosis including massive edema at the time therapy was started, irrespective of how much time had elapsed after their first episode. This is not in our favor.

DR. RILEY: No, because some of those may have been going to get well on their own hook anyway.

DR. LANGE: Very well, but I have said that no further fatality would have occurred in them, because we have assumed that, after the 56th month, no further fatality would have occurred in the control group, a somewhat improbable assumption.

DR. RILEY: But I am referring to your own cases. You see, when you get a patient who has been 5 years with his disease, the chances are he is going to live anyway. Meanwhile, there have been a lot of cases you have not seen, who have died in the first 5 years of their disease, so that, by including those you first saw such a long time after onset --

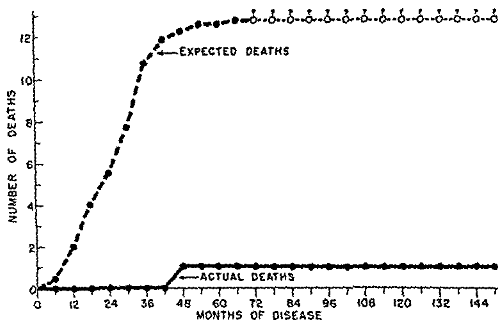


Fig. 81. Graphic representation of data from Table 43. The graph illustrates a comparison between expected versus actual deaths. Note that at 65 months the number of expected deaths would have been 12.8, the actual deaths observed were 1.

DR. LANGE: That is not necessarily so. Every case which comes under our observation was treated irrespective of duration of disease.

DR. BAXTER: Dr. Lange, does this include all the patients you have seen with significant proteinuria?

DR. LANGE: Yes, proteinuria and edema, the full-blown picture of nephrosis, with or without underlying nephritic components.

DR. BAXTER: It seems to me that is the big question, because all of our patients have had red cells in the urine. Where do you draw the line? That has always puzzled me.

DR. LANGE: Many of them had red cells in the urine, and quite a number of them had elevated BUNs when they were admitted. None of them, since 1951, was exempt from this group; no selection was made.

DR. METCOFF: May I get one point clear? The chi-square which you have drawn is really after 65 months. It refers to performance at the end of --

DR. LANGE: No, after the 148 months.

DR. METCOFF: It was my understanding of the technical manipulation in chi-square that one had to have at least 5 entries in each cell before what the test was designed for would be valid.

DR. LANGE: What do you mean? Five degrees of freedom?

DR. METCOFF: No; you can have one degree of freedom. But you have to have at least 5 individuals in each category in order to avoid exaggerating the χ^2. Your table might be all right at 65 months, but certainly would not be all right when you get down to only 3 individuals.

DR. LANGE: But this is an additive number of deaths in the treated group as compared to an additive number of expected deaths based on the deaths in the control group. In addition, as one can easily see from Table 43 the statistical result would have been exactly the same had we drawn our dividing line at 65 months rather than at 149 months. The number of expected deaths would have been 12.8. The number of actual deaths, one, with a chi-square value of .001. Dr. Gumbel, of the School of Engineering at Columbia University agreed to the statistical methods.

Figure 81 represents the same data in graphic form, showing the expected deaths compared to the actual deaths.

I wish to state one more fact. We had 3 cases in whom we were unable to induce diuresis; in one of them five attempts with different steroids in large doses were unsuccessful and in the others four attempts were unsuccessful. We have diuresed 3 of these cases (one adult, two children) with intravenous ACTH. We gave 30 units of ACTH intravenously in 600 to 1000 cc. of 5 per cent glucose per day, over 8 hours, and

continued that for 10 days, during which time these children did not give us too much difficulty, provided one can find a vein. Why they did not diurese on large doses of ACTH i.m. before, whether there was a local trapping, inactivation, or something happened to the ACTH in the tissue, I do not know.

DR. GOODMAN: Did they have a decrease in proteinuria as well?

DR. LANGE: Yes, proteinuria decreased; but none of the 3 is free of proteinuria; they have a 1+ proteinuria.

CHAIRMAN BARNETT: Dr. McCrory!

D. Dr. Wallace McCrory

DR. McCRORY (Children's Hospital, Philadelphia): Our concept of the orientation of therapy at the moment, established or not, is that steroid therapy possesses the capacity to suppress the pathologic lesion of the nephrotic syndrome.

If this is true, it is rational to give steroid therapy until the clinical and laboratory evidences which we take to assess the activity of this process indicate remission or improvement.

This is not to say we are successful with every patient but I do want to make it clear that we try intensive treatment in all patients. We did state, a couple of years ago, when treating for the arbitrary period of 28 days, that we found no patients who had not diuresed in this time who in our experience, subsequently diuresed after a longer period of therapy.

I think that this observation has been interpreted by some to mean that we feel therapy should cease after a period of 28 days.

We have now personally observed and heard, by coming back to this meeting yearly, of diuresis occurring in patients treated for two or three or four months.

I can put this most simply by telling you what we would do, regardless of whether we have a patient sent to us after three years of unsuccessful therapy elsewhere or early in the course of his disease.

After establishing the diagnosis of nephrosis and making certain that the patient is free of any active infection, we start them on steroid. At the moment we use Meticorten, because we seem to have fewer electrolyte problems.

We start children on a dosage of 1 mg. per pound per day, actual weight. We employ some dietary salt restriction. By giving patients a diet containing not more than a half-gram of salt a day, we have found that the increase in weight which we usually observe in children with edema early in their course can, by and large, be limited, if not prevented.

Diuresis is often seen after one to two weeks of such therapy. We do not alter the dosage of steroid with the occurrence of diuresis. While this is therapeutically desirable, it is not the aim of therapy. We continue treatment if possible not only until proteinuria ceases but until serum protein concentrations and sedimentation rate have returned to normal. When this occurs, the dosage of steroid is gradually tapered and stopped. Further steroid treatment is dependent on the course of subsequent follow-ups. If proteinuria recurs and persists in the absence of intercurrent infection, the patient is re-treated.

In many instances, cessation of proteinuria has not occurred after a number of weeks of such therapy even though serum protein concentrations improve. We place such patients that are edema-free on intermittent treatment in place of continuous therapy. Intermittent steroid therapy consists of having them take an amount of steroid similar to their previous daily dosage for only four consecutive days out of each week. This "intermittent" administration is continued until they seem stabilized or show complete remission. If proteinuria increases or edema recurs, continuous administration is re-instituted. On this regimen the great majority of patients can be maintained in sufficiently good general health to lead an almost normal life. The optimum duration of steroid therapy has yet to be demonstrated. One may be guided by observing the effects of reduction or cessation of steroid therapy on the course and laboratory signs of the disease.

Some patients fail to show any improvement after 3-4 weeks of continuous steroid therapy. We have been able to induce partial or complete loss of edema in some of these patients by a series of daily infusions of albumin. The high protein feeding regimen suggested by Blainey[17] has also proven useful in some of these patients in controlling edema.

We have continued this dosage of steroid in some unresponsive patients for as long as three or four months after hearing that others have done the same thing and in a few instances, gradual loss of edema has been seen.

DR. HEYMANN: What was the dose of meticorten used in these patients?

DR. McCRORY: It would still be a mg. per pound of meticorten daily or cortisone, 4-5 mg. per pound per day.

CHAIRMAN BARNETT: And this is a daily maintenance dose?

DR. McCRORY: By maintenance, I mean a short period of administration alternating with a few days of no steroid, for instance 1 mg./lb./day of meticorten for four days followed by three days of no steroid - this is that we call "maintenance." This type of therapy may maintain improvement but it does not induce remission.

DR. GRIBETZ: What is the longest you have had to continue this therapy on a child who has been symptom-free except for proteinuria?

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[17] Blainey, J. D., High Protein Diets in the Treatment of the Nephrotic Syndrome, Clin. Sci. 13: 567, 1954.

DR. RAPOPORT: Three years.

DR. McCRORY: We prefer to have patients on long term treatment on an intermittent regimen. We have done this because we have felt it allows one to stop steroid treatment abruptly if necessary because of viral infections or other complications that are best handled without steroid therapy. In our experience, the children on the intermittent maintenance dosage can tolerate withdrawal without going into adrenal insufficiency while the children on continuous therapy really pose a major problem in this regard.

When patients have been edema-free for three to six months, we will attempt to reduce gradually the amount we give for maintenance, finally discontinuing steroid treatment if they seem stabilized.

CHAIRMAN BARNETT: I think, as I said before, somewhere in between these two types of approach to therapy is what most of us are doing.

DR. RAPOPORT. May I interrupt at this point? I think the orientation ought not to be on a scheme schedule. One has certain objectives which one wants to achieve; how one plays the variation on this theme I do not think is very important because, actually, the objectives which we want to achieve can be seen and measured. We still cannot look at the fundamental lesion; and nobody is doing renal biopsies every day, or every week. So all we can do is to say that these are the criteria for remission; somehow, we manage to achieve this. We have to tailor-make this suit to fit each individual child. When one gets to the point of tapering off - and tapering off actually consists of gently withdrawing one's finger from the hole in the dike, to see if it is still leaking - how to taper off is one's own business, whether to do this three days a week, four days a week, a big dose one day, a small dose the next day, a dose every other day, etc; at any rate we just sort of gradually step backward until we get down to no steroid. With some children, we can do this quickly; in some, if we do it quickly and proteinuria recurs, we are a little more cautious the next time about the withdrawal, and stretch out the tapering period.

In this sense, we are not adhering to any scheme. In the beginning, when this first started, we very pig-headedly stayed with the scheme; we said: "Well, you get free of edema, this is it; we quit. We will treat you again when you get edema." If we had known more then, we would probably have done it differently. But I think we have to have a kind of fluidity about treatment, which considers the patient and not any fixed scheme.

To try to compare this, we do not have standards of reference right now; I do not think any of these complications take into account a tremendous number of variables which I am sure most of us are aware of, and which will not show on the chart. Some of these are variations of personalities, of the person doing the treatment, of the kind of patients we have. There are all sorts of intangibles which enter into this.

Perhaps if we all agreed on a set of objectives -- The surgeons say, "We want a five-year evaluation, not cure, but a five-year 'you are still alive at the end of an operation'."

The American Heart Association says there are criteria for remission of rheumatic-heart disease. They did not say that the last lesion must be gone. I think we all ought to agree about what the objective of treatment is. I do not care how we do this. One of us thinks we can handle the problem a little differently than the next one, in terms of not wanting to overtreat or undertreat - this is another matter - but we ought to agree on our objective.

CHAIRMAN BARNETT: I agree with you; and I think this is what most of us are doing. The one thing, however, which I think we must face frankly is that Dr. Lange's results which he has shown again today, in which there is an arbitrary period of long-term therapy which is not related to the things you are talking about, are based on the theoretical considerations which I think reflect his belief about the pathogenesis of this disease. This is the only group of patients who, I think, are being treated essentially very differently from the way the rest of us are treating, with all the variations the rest of us are using. We are faced with the fact that, to my knowledge, no group of patients, even of this size, over a period of 6 years, has done as well as his group. I am not asking, you know, for any rigid kind of treatment scheme. I would agree with you that I think what we are faced with is a philosophy of treatment -- The regimen which Dr. Lange has proposed, is philosophically a different regimen, which does not depend upon varying treatment, upon those manifestations which you were talking about, Dr. Rapoport.

I suppose, if there is any real difference and any purpose in the controlled study, it would be (which Dr. Lange has been asking for for a long time) a comparison between his regimen and a regimen which is based essentially on what you are talking about, which is what most of the rest of us are doing.

DR. RAPOPORT: I do not know whether you can resolve this until many years have gone by.

DR. HEYMANN: Don't you think you could have the same results as Dr. Lange, if you had 6 years of experience with intensive treatment right from onset, instead of only 2 or 3 years?

DR. McCRORY: There is one point I would like to hear a little discussion about.

If steroid therapy is beneficial, it may be important to study the course in patients adequately treated with respect to the time interval between onset of the disease and the initiation of a regimen of steroid treatment.

I think this makes the figures we have very difficult, because we exercise no selection; we take all patients. The impression we have gotten by looking over the figures is that they get rosier in terms of the response of patients if you see only those children who respond very quickly after recognition of their disease. Would it not be possible, in getting figures on survival, for one to look over a huge group and see whether there is benefit from the use of a reasonable scheme versus no regimen early in the course of the disease?

CHAIRMAN BARNETT. Dr. Kramer, could you just tell us briefly about your experience?

DR. METCOFF: I wonder if I might make one point while we are waiting for Dr. Kramer's slides. That is, aside from the scheduling and the observation of a system of therapy, it might be worthwhile if we tried to devise some systematic scheme of observation that all of us might adhere to so that, irrespective of what the therapeutic schedule would be, we might have similar quantitative observations at similar points in time, which would make assessment or comparison of different schedules reasonable.

CHAIRMAN BARNETT: May I just say I will not cut Dr. Kramer off in the middle of a sentence at twelve o'clock; but we will cut somebody off in the middle of a sentence at twelve-fifteen.

E. Dr. Benjamin Kramer

DR. KRAMER (Jewish Hospital, Brooklyn, New York): Because of limitation of time, I am going to eliminate the preliminaries and report on the value of the use of a new synthetic steroid in the treatment of children with nephrosis. The study was carried out by Drs. Leon Hellman, Barnett Zumoff, Norman Kretchmer and myself.

This new synthetic steroid hormone possesses certain biochemical properties differing from, and more desirable than, those of other compounds presently in use.

The structure of this new synthetic steroid is 16-alpha-hydroxy, delta-1, 9-alpha-fluoro-hydrocortisone, which is known also under the name of Triamcinolone. It is basically hydrocortisone modified to contain the delta-1 bond characteristic of prednisone, the fluorine atom at the 9 position characteristic of fluorohydrocortisone, and in addition, a hydroxyl group in the alpha orientation on the 16 carbon atom. The parent compound, delta-1-fluorohydrocortisone, is a potent salt retainer, but the insertion of the 16-alpha hydroxyl group serves to abolish the sodium retaining properties.

Triamcinolone causes no sodium retention, in fact, most normal non-edematous patients with intact adrenals show sodium loss during the first few days of its administration, usually accompanied by equivalent weight loss. Normal patients do not continue to lose sodium after the first few days, but come into sodium balance. At normal therapeutic dose levels there is no potassium loss induced by this steroid, and only minimal losses of nitrogen and calcium. There is no blood pressure elevation induced by Triamcinolone. There also seems to be a decreased incidence of other undesirable side effects, including gastric ulcers.

The use of this compound was explored in a series of 14 children with the nephrotic syndrome, comprising 4 females ranging in age from 1 to 9 years and 10 males ranging in age from 2 to 5 years. Of the 14 patients only 2 had any signs of renal insufficiency; in one child this was manifested by elevated blood pressure only, whereas the other child had both azotemia and elevated blood pressure. The other 12 members of the series had the nephrotic syndrome in its characteristic form, without any stigmata of renal insufficiency. The duration of the nephrotic syndrome in these patients had been from 2 weeks to 3 years.

In all, 20 complete courses of treatment were given to these 14 patients. By our criteria, a complete course of treatment was one that consisted of the administration

of 20 mg. per day of Triamcinolone in 4 divided oral doses of 5 mg. each for a minimum of 30 days, and usually extending from 30-45 days. Six of the 20 courses of treatment represented re-treatment of patients, 2 of whom had received inadequate initial courses and the other 4 were patients who had relapsed after a previously complete course. This regimen of 20 mg. per day for 30-45 days induced 12 complete remissions out of the 20 courses of treatment given. A complete remission was one in which the urine findings were indistinguishable from those of a normal individual, and all the abnormal chemical findings had returned to normal. There were 6 partial remissions, which meant that a diuresis had been induced, and although there was improvement of the abnormal blood and urine chemical findings, they failed to return completely to normal. Two courses were failures, in the sense that no diuresis occurred and there was no change in either the blood or the urine findings. The 2 courses resulting in failures occurred in the 2 patients who had prior signs of renal insufficiency. It appeared that the patients who had had the nephrotic syndrome for the shortest period of time were the ones with the greatest likelihood of having a complete remission. This agrees with the experience of other workers in the field.

Complete remissions were induced in 9 patients. Of these 9, 5 have remained in complete remission. Four of the 9 relapsed at approximately 3-4 months after the conclusion of treatment. These 4 were re-treated, a second complete remission was induced in 3 of them, and a partial remission in the fourth. Thus, at the present time 8 of the original 14 patients are in complete remission; the remissions have lasted for 2 weeks to 15 months since the conclusion of treatment with Triamcinolone.

With reference to prior therapy with either ACTH or other steroids, of the 9 patients who had a complete remission on Triamcinolone, 3 previously had complete remissions on other forms of steroid therapy. One patient who had a complete remission on Triamcinolone had failed to respond to other forms of steroid therapy. Two of the 9 patients had no prior treatment. In the partial remission group only 1 out of the 4 patients had previously had a complete remission on other steroid therapy, 3 of the 4 had had partial remissions on other forms of steroid therapy.

This preliminary report of the use of the new 16-alpha hydroxy substituted steroid, Triamcinolone, in the treatment of nephrosis has yielded results which are similar to those obtained with ACTH, cortisone, or prednisone. The results are of interest for two reasons: 1) They demonstrate that a new class of steroid compounds can induce remissions in the nephrotic syndrome; it is possible, although there is no evidence available as yet, that patients who fail to respond to one or another of the presently available steroids, may have remissions when treated with Triamcinolone, and it certainly would be worthwhile to explore the effects of Triamcinolone in patients resistant to other forms of steroid therapy. 2) The other interesting aspect concerns the relative inertness of this compound in producing changes in either sodium or potassium metabolism in the normal individual. These properties of the steroid point up the fact that neither the exhibition of a sodium-retaining action nor the production of negative potassium balance are prerequisites for the functioning of a steroid in the effective treatment of nephrosis.

CHAIRMAN BARNETT: Thank you, Dr. Kramer.

Are there a few questions or comments?

DR. HEYMANN: I would like to ask Dr. Kramer whether spontaneous fractures or osteoporosis were seen when Triamcinolone was used over similarly long periods, as we have observed in two patients who were treated over more than seven months' period, one with cortisone, the other with Meticorten?

DR. KRAMER: As far as I can recall, in our discussions with Dr. Talman, who used it over a long period of time, he experienced that complication.

DR. JANEWAY: You say you have seen occurrence of that. Has anyone here seen an ulcer in a child?

DR. RAPOPORT: We have one questionable one.

DR. JANEWAY: It certainly is rare.

DR. McCRORY: I have heard of a child, fortunately treated elsewhere, who had a duodenal perforation.

DR. METCOFF: We had one such experience.

DR. JANEWAY: We had one, but not in a nephrotic.

DR. GRIBETZ: Was yours one you had on a continuous daily dosage?

DR. JANEWAY: Yes.

DR. GRIBETZ. For how long?

DR. JANEWAY: I think the child had been on steroid therapy for 8 or 9 months.

DR. GRIBETZ. It seems as if there might be a difference between continuous maintenance steroid therapy and the intermittent three-day-a-week regimens. At least, the growth depressing effect is not seen [18]. We have analyzed the data of our children on intermittent therapy to see whether growth is depressed. It does not appear to be as was the case with prolonged continuous therapy.

CHAIRMAN BARNETT I would like to conclude this part with the suggestion that we ask Dr. Riley, who has had great interest in this kind of evaluation of data, and who would like to continue it, if he would be willing to consider our discussions here this morning in terms of what sort of analysis or what proposals for collaborative observations might be made; and after giving this thought, he prepare a letter which would embody his thoughts and suggestions, to be sent to each of us.

Would you be willing to do this, Con? We can give you a list of the participants, and also other large clinics not represented here.

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[18] Blodgett, F. M., Burgin, L., Iezzone, D., Gribetz, D. and Talbot, N.B., Effects of prolonged cortisone therapy on the statural growth, skeletal maturation and metabolic status of children. N. E. J. Med., 254: 636, 1956.

(Dr. Riley nods his head in agreement.)

CHAIRMAN BARNETT: Thank you.

DR. SCHREINER: I would like to raise a question about the criteria for remission, which we take as no substantial proteinuria and normal chemistries.

We have biopsied people who have had remissions of a year or two years, and found a percentage of the glomerular population fibrosed.

I think you can say one thing about proteinuria; at least there is blood perfusing the glomerulus. Proteinuria may cease; but possibly treatment may do it by fibrosing the glomerulus as well as by improving the so-called nephrotic lesion. I wonder whether we may not confuse some of our statistics by setting this as the ultimate criterion for remission?

CHAIRMAN BARNETT: I would like to echo Dr. Metcoff's thanks to Dr. Baxter and to Dr. Goodman and to all the participants here. I would also like to thank again the National Nephrosis Foundation, whose President, Dr. Kaessler, has been with us during part of this meeting and most of the other meetings during the last few years. The Foundation, led by Dr. Kaessler, has made it financially possible for the transactions of this meeting to be transcribed and to be published.

I think most of you know Dr. Kaessler; he is sitting over here in the second chair.

DR. McCRORY: What about next year?

CHAIRMAN BARNETT: It is not decided. The plan was that we would meet here this year, then probably in New York next year, at the Babies' Hospital; but this is not firmly decided.

(The meeting adjourned at twelve-fifteen o'clock.)

ACKNOWLEDGMENTS

We wish to express our appreciation to the following journals for editorial permission to reproduce the figures indicated:

Figures 22, 66, 67, 68, 71 a and b, 72
American Journal of Physiology

Figures 18 to 23
Bulletin Johns Hopkins Hospital

Figure 24
The Deutsche Medizinische Wocheschrift

Figure 42
Federation Proceedings (The American Physiological Society)

Tables 11 to 13
Journal of Clinical Investigation

Figure 58
Laboratory Investigation

Figures 73 and 74
New England Journal of Medicine

Figures 25 and 26 and Table 7
Zeitschrift fur die Gesamte Experimentelle Medizin